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(54) Title: INHIBITORS OF REGULATORY PATHWAYS (57) Abstract Methods are provided for screening for potential inhibitors of bacterial, or other microbial, global pathogenesis gene regulators and other gene regulators. Methods are also provided for treating microbial (e.g., bacterial) infections using such inhibitors. Also included are pharmaceutical compositions containing such inhibitors. The screening methods involve detecting whether the activity of a global pathogenesis gene regulator is altered in the presence of a test compound.		

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DESCRIPTION

INHIBITORS OF REGULATORY PATHWAYS

Background of the Invention

This invention is related to the fields of antimicrobial agents and the treatment of microbial infections. It is, in addition, concerned with methods
5 for identifying antimicrobial agents and agents which facilitate the action of antimicrobial agents.

None of the information presented below is admitted to be prior art to the pending claims, but is provided only to aid the understanding of the reader.

10 The traditional approach to treatment of microbial infections has largely been to treat with antibiotics which either kill the microbes (microcidal) or inhibit microbial growth. These antibiotics exert their antimicrobial action both in culture (in vitro),
15 and in an infection (in vivo). Extensive screening by the pharmaceutical industry in the last fifty years for bactericidal and bacteriostatic compounds has led to the discovery and development of large numbers of antibiotics, most of which are members of a much smaller

number of structural or functional classes. Examples of those classes of antibiotics are the β -lactams (which include the penicillins and cephalosporins), aminoglycosides, and glycopeptides.

5 However, an increasingly serious problem is the spread of broad antibiotic resistance, both geographically and in different microbial species. Antibiotic resistance is particularly notable in bacteria. Such bacterial resistance to an antibiotic(s)
10 may be due to any of a number of mechanisms. For β -lactam resistance, an important mode is the production of β -lactamases. Other mechanisms which result in drug resistance include the development of altered antibiotic targets and reduced cellular uptake of the drug.

15 An example of the development of antibiotic resistance is the appearance of methicillin resistance in *Staphylococcus aureus*. Methicillin is a penicillinase-stable β -lactam antibiotic often used for the treatment of penicillinase-producing strains of
20 *Staphylococcus aureus*. However, methicillin-resistant *S. aureus* (MRSA) have acquired a methicillin-insensitive cellular target which allows bacteria to grow in the presence of the drug, and the incidence of MRSA infections has become a serious problem (Chambers, *Clin. Microb. Rev.* 1:173-186, 1988; De Lencastre et al., *J. Antimicrob. Chemother.* 33:7-24, 1994). The current
25 average incidence of MRSA in some large hospitals in the USA increased from 8% in 1986 to 40% in 1992, and there are MRSA strains which are susceptible to only a single
30 class of clinically available antibiotics, the

glycopeptides. There is a need for the discovery of new efficient anti-MRSA drugs before resistance to glycopeptide antibiotics develops in multi-resistant MRSA strains.

5 The problems associated with antibiotic resistant bacteria are not limited to *S. aureus*, but are present in a large number of bacterial pathogens. Therefore, there is a need for the development of new types of antibacterial agents, including ones directed
10 'to new targets. Such new antibacterial agents will not only reduce the problems associated with treating infections involving resistant bacteria, but can also provide additional therapeutic options even for treating bacteria which are still susceptible to currently
15 available antibacterial agents.

 One approach to developing such new antibacterial agents is to target bacterial pathogenesis. The bacterial products related to pathogenesis are often termed "virulence factors".
20 Virulence factors are those biological molecules produced by a pathogenic bacterium that are essential for survival in the host organism but are not necessarily essential *in vitro* (where survival is meant to connote entry, attachment, evasion of host immune system,
25 nutrient acquisition, and any other molecular processes necessary for adaptation to the host environment). Since most screening for novel antibiotics has been performed *in vitro*, virulence factors remain unexploited targets for antibiotic discovery screens. Based on
30 estimates from other pathogenic organisms, the number of

such virulence genes in *Staphylococcus aureus* is estimated to be 50-100 (see, Groisman and Ochman, *Trends in Microbiol. Sci.* 2:289-294 (1994); Muhldorfer and Hacker, *Microb. Pathogenesis* 16:171-181 (1994)).

5

Summary of the Invention

This invention provides a novel approach to treating microbial pathogenesis by the administration of small molecules (compounds) which alter the functioning of a microbial global regulator of pathogenes. This invention recognizes that reducing or eliminating the production of exoproteins, toxins, or other factors related to bacterial pathogenesis by affecting the regulation of their synthesis and secretion, can greatly alter the course and effects of a bacterial infection.

15 This treatment approach is distinct from most prior antimicrobial treatments which attempted to either kill the microbial cells, or directly prevent them from reproducing. The antimicrobial action of the compounds of such antimicrobial treatments is exerted both in vivo, in an infection, and in vitro, in a culture, unless some specific compensating factor(s) is provided which allows survival or growth in the presence of the antimicrobial agent. In contrast, this invention is directed at the global regulation of microbial factors

20 which are involved in the pathogenesis process, but are not necessarily essential for microbial survival or growth in vitro.

Such pathogenesis factors are associated with specific microbial genes, which may encode a variety of

different types of pathogenesis-related products. Such products may include, e.g., specific protein toxins and regulatory molecules which affect the production of other molecules such as toxins. The *agr* locus of *Staphylococcus aureus* is an example of a set of genes which encodes regulatory molecules which control the production of a large number of toxins and exoproteins, and enzymes. In particular, transcription of RNAIII from the P3 promoter is essential for the *agr* effect on the related exoproteins (see Detailed Description below), and is thus identified as a global pathogenesis gene regulator. Thus, inhibition of the production of RNAIII inhibits the *agr* effect, which includes both up-regulation of certain products and down-regulation of other products. However, inhibition of other global regulators of pathogenes, whether in *Staphylococcus aureus* or in other bacterial species, has similar potential.

In this invention, small molecules have been identified that inhibit the naturally occurring, growth dependent induction of the P3-driven RNAIII and those downstream virulence factors under its influence. Such compounds, termed antipathogenics, may show some growth inhibitory activity at higher concentrations, but also show antipathogenic behavior at sub-MIC levels.

(Antipathogenics refers to compounds which reduce the pathogenesis-related effects of one or more pathogenesis factors.) Therefore, treatment with such molecules has effects such as attenuating host inflammatory response, decreasing load of bacterial toxins, disfavoring

colonization of the host by the bacteria, allowing the host to clear the infection, or potentiating the effect of traditional antibiotic drugs by weakening the bacteria or relocating them to a drug- or host factor-accessible compartment.

Therefore, in a first aspect, this invention provides methods of treating a bacterial infection of an animal, preferably of a mammal, specifically including in a human, by administering a compound which alters the activity of a global regulator of pathogenes. In a preferred embodiment, this is accomplished by inhibiting the level of activity of the global regulator, such as by inhibiting expression of that regulator by the administered compound. However, a compound which inhibits expression of a global regulator (or otherwise affects the level of activity of the regulator) need not act directly on that specific gene (or molecule). For instance, such an inhibitor can act at an upstream regulator, which directly or indirectly regulates expression of the global regulator. In a preferred embodiment, the compound inhibits the expression of RNAIII, or is active against the *agr*, *xpr*, *sae*, or *sar* genes or a homolog thereof.

In this context, the term "treating" refers to administering a pharmaceutical composition for prophylactic and/or therapeutic purposes. The term "prophylactic treatment" refers to treating a patient who is not yet infected but is susceptible to or otherwise at risk of a particular infection. The term "therapeutic treatment" refers to administering

treatment to a patient already suffering from an infection.

The term "bacterial infection" refers to the invasion of the host animal (e.g., mammal) by pathogenic bacteria. This includes the excessive growth of bacteria which are normally present in or on the body of a mammal, but more generally, a bacterial infection can be any situation in which the presence of a bacterial population(s) is damaging to a host mammal. Thus, a mammal is suffering from a bacterial infection when excessive numbers of a bacterial population are present in or on a mammal's body, or when the effects of the presence of a bacterial population(s) is damaging the cells or other tissue of a mammal.

The terms "administering" and "administration" refer to a method of giving a dosage of an antibacterial pharmaceutical composition to a mammal where the method is, e.g., topical, oral, intravenous, transdermal, intraperitoneal, intramuscular, or intrathecal. The preferred method of administration can vary depending on various factors, e.g., the components of the pharmaceutical composition, the site of the potential or actual bacterial infection, the bacterium involved, and the severity of an actual bacterial infection.

By "comprising" it is meant including, but not limited to, whatever follows the word "comprising." Thus, use of the term "comprising" indicates that the listed elements are required or mandatory, but that other elements are optional and may or may not be present. By "consisting of" is meant including, and

limited to whatever follows the phrase "consisting of." Thus, the phrase "consisting of" indicates that the listed elements are required or mandatory, and that no other elements may be present. By "consisting essentially of" is meant including any elements listed after the phrase, and limited to other elements that do not interfere with or contribute to the activity or action specified in the disclosure for the listed elements. Thus, the phrase "consisting essentially of" indicates that the listed elements are required or mandatory, but that other elements are optional and may or may not be present depending upon whether or not they affect the activity or action of the listed elements.

The term "mammal" refers to any organism of the Class Mammalia of higher vertebrates that nourish their young with milk secreted by mammary glands, e.g., mouse, rat, and, in particular, human, dog, and cat.

In the context of treating a bacterial infection a "therapeutically effective amount" or "pharmaceutically effective amount" indicates an amount of an antibacterial agent, e.g., as disclosed for this invention, which has a therapeutic effect. This generally refers to the inhibition, to some extent, of the normal cellular functioning of bacterial cells causing or contributing to a bacterial infection. The dose of antibacterial agent which is useful as a treatment is a "therapeutically effective amount." Thus, as used herein, a therapeutically effective amount means an amount of an antibacterial agent which produces the desired therapeutic effect as judged by clinical

trial results and/or animal models of infection. This amount can be routinely determined by one skilled in the art and will vary depending upon several factors, such as the particular bacterial strain involved and the
5 particular antibacterial agent used. This amount can further depend on the patient's height, weight, sex, age, and renal and liver function or other medical history. For these purposes, a therapeutic effect is one which relieves to some extent one or more of the
10 symptoms of the infection and includes curing an infection.

As used in this disclosure the term "pathogenesis factor" or "virulence factor" refers to a molecule produced by an infecting organism which has a
15 significant function in the bacterial infection process. This includes molecules which are involved in the adaptation of the bacteria to an animal (e.g., mammalian host), establishment of a bacterial infection, in the maintenance of a bacterial infection, and in producing
20 the damaging effects of the infection to the host organism. Further, the term includes molecules which act directly on host tissue, as well as molecules which regulate the activity or production of other pathogenesis factors.

25 In *Staphylococcus aureus*, an "agr-related pathogenesis factor" is a molecule which is significantly linked with the expression of the operons of the *agr* locus. Thus, the term includes the specific toxins, proteases, and other pathogenesis factors
30 regulated by the products of the *agr* locus, as well as

both upstream and downstream regulatory molecules.

Another related term "pathogenesis genes" or "pathogenes" refers to a bacterial gene which encodes a pathogenesis factor, which includes genes which directly
5 encode products such as proteases, as well as biosynthetic genes which encode a product which is directly involved in the synthesis of non-polypeptide molecules involved in pathogenesis. It further includes genes which encode regulatory molecules which affect the
10 level of production of other molecules.

Some such regulatory molecules are termed "global regulators." While a regulatory molecule controls the level of expression of at least one other bacterial gene, to be termed a global regulator such a
15 molecule should coordinately regulate the expression of several linked or unlinked genes to achieve a particular physiological adaptation of functional outcome. This does not mean that the global regulator exerts exclusive control over the level of expression of those other
20 genes, but rather means that a change in the level of activity of the global regulator will significantly alter the level of expression of at least three other genes. A clear example of such a global regulator is the RNAIII transcript, which is transcribed from the P3
25 promoter of the *agr* locus in *Staphylococcus aureus*, and which affects the level of expression of numerous products, some of which are specified in the Description of the Preferred Embodiments below. Since numerous of the products regulated by the *agr* locus are pathogenesis
30 factors, the *agr* locus is a global regulator of

pathogenesis genes.

In the context of global regulators of pathogenesis genes, the term "homolog" refers to gene sequences from different bacterial strains or species which have significantly related nucleotide sequences, and consequently gene products which have significantly related nucleotide or amino acid sequences. Preferably, homologous gene sequences will have at least 50% sequence identity (as defined by the maximal base match in a computer-generated alignment of two nucleic acid sequences), more preferably at least 60%, and most preferably at least 80%. For polypeptide gene products of such homologous genes, generally the gene products also exhibit a significant degree of amino acid sequence identity. Thus, for such polypeptide products of homologous genes, the amino acid sequences have at least 25% sequence identity over a sequence of 100 or more amino acids, more preferably at least 40%, still more preferably at least 60%, and most preferably at least 80%. In addition, in the present context, the products of the homologous gene sequences are also involved in regulation of a cellular response.

In a further aspect, this invention provides methods of treating an infection involving a bacterium (e.g., *S. aureus*) by administering a compound which inhibits one or more pathogenesis factors modulated by *agr*, *xpr*, *sar*, or *sae*. Usually, but not necessarily, this involves reducing the production of those factors.

The term "modulated" means that the level of a pathogenesis factor in the in vivo environment can be

altered by changes in the presence or concentration of a particular gene product. For example, the *agr*-related pathogenesis factors are modulated by RNAIII.

In a related aspect this invention also
5 provides a method for prophylactic treatment of a mammal, in particular a human, in order to prevent a bacterial infection. Such treatment comprises administering an inhibitor of a global regulator to the mammal. Preferably such treatment would be used when
10 the patient is at risk of contracting or developing a bacterial infection. Particular embodiments of this method are as described above for the method of treating a bacterial infection. Such a prophylactic treatment method may have particular benefit, for example, for
15 treating patients prior to surgical operations.

In another related aspect, this invention provides pharmaceutical compositions having an inhibitor of a global pathogene regulator and a pharmaceutically acceptable carrier.

20 The specific screening hits described as Compounds 1-18 (Figs. 6-8) are more generally described by the Structures 1-14, including the narrower descriptions of Structures 1A, 2A, 2B, 3A, 4A, 5A, 6A, 7A, 8A, 9A, 10A, 11A, 12A, 13A, and 14A (as shown in
25 Figs. 9-12), which also describe related compounds. Active related compounds can be obtained, for example, as commercially available analogs of previously identified hits, by synthesis, by modification of identified active compounds, and by identification of
30 new screening hits. Thus, in preferred embodiments of

the therapeutic compositions, methods of treating a bacterial infection, and methods of prophylactic treatment described herein, the inhibitor is an active compound described by one of Structures 1-2 and 4-14 or one of the corresponding narrower structure descriptions, Structures 1A, 2A, 2B, 4A, 5A, 6A, 7A, 8A, 9A, 10A, 11A, 12A, 13A, and 14A. For each of the general structures, the substituent groups (R or X groups) are as described in the Detailed Description below.

Likewise, in another related aspect, this invention provides pharmaceutical compositions having an inhibitor of a regulator related to *agr*, *sar*, *sae*, or *xpr*, or of one or more pathogenesis factors modulated by *agr*, *sar*, *sae*, or *xpr*, and a pharmaceutically acceptable carrier.

In a further aspect the invention provides screening methods for potential inhibitors of global regulators of bacterial pathogenes. In preferred embodiments, the amount of translation product resulting from a globally regulated promoter region/reporter gene hybrid construct is determined in the presence and absence of potential inhibitors. The DNA construct is incorporated into a bacterial cell which can induce transcription from the pathogenesis gene promoter in response to an environmental signal. A potential inhibitor is then a compound which reduces the level of, or eliminates, transcription of the hybrid DNA, and so eliminates, or reduces the amount of the translation product. In an embodiment where the reporter gene is a

β -lactamase gene, the presence of the translation product can be detected by the ability of the bacterial cell culture to produce color change in chromogenic β -lactamase substrate, such as nitrocefin, or by selection
5 and growth of cells on a cleavable β -lactam-containing medium.

Such a potential inhibitor may act at any point of a regulatory sequence which is either upstream of or at the specific promoter. In the case where the P3
10 promoter as described above is used, such upstream elements include at least the products of the *agrABCD* genes of the P2 operon but are not limited to those elements. Inhibitors that block the *agr*-mediated global regulation will prevent induction of the *Agr*-P3
15 promoter, as well as downstream toxins and degradative enzymes.

In addition, since the above methods of screening are suitable for both known and unknown compounds and compound libraries, the invention also
20 provides novel inhibitors for global regulators of pathogenes, such as RNAIII, or of components of the *agr* effect in *S. aureus* besides RNAIII.

The identification of novel targets and therapeutic approaches provides a method for preparing
25 therapeutic agents active on global regulators of pathogenesis genes. Thus, in a further aspect, the invention provides methods of making an antibacterial agent. The methods involve screening for the agent by measuring the ability of the agent to alter the level of
30 activity of a global regulator of pathogenesis genes,

and synthesizing the therapeutic agent in an amount sufficient to provide the agent in a therapeutically effective amount to a patient. In a preferred embodiment, the screening involves detecting the amount
5 of transcriptional or translational product from a hybrid DNA construct inserted in a cell, for cells grown in the presence and absence of the agent. The construct includes a regulatory region of a gene encoding a global regulator of pathogenesis genes, transcriptionally
10 linked with a reporter gene.

Also in preferred embodiments, the global regulator is from a Staphylococcal strain, for example, the regulator can be encoded by a gene of the *agr* locus, *xpr*, *sar*, or *sae*. In particular preferred embodiments,
15 the agent has a structure which is one of the structures described herein, e.g., Structures 1, 2, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, or 14. Further, the method can include the step of adding a pharmaceutically acceptable carrier to the agent.

20 Other features and advantages of this invention will be apparent from the following description of the preferred embodiments thereof, and from the claims.

Brief Description of the Figures

FIGURE 1. Schematic representation of the *Agr* operon
25 and related pathways. The *Agr* operon is comprised of three promoters driving five genes (*agr ABCD*, and *hld*) as well as one regulatory RNA molecule (*RNAIII*) whose coding sequence overlaps that of *hld*. *AgrC/A* are thought to constitute a two-component regulatory system,

where AgrC is the putative sensor/kinase and AgrA is the response regulator.

In response to environmental or endogenously produced signals like the AgrBD-derived octapeptide, this two-component system transduces a signal that results in the transcription of promoters P2 and P3. The RNAIII produced from the P3 promoter has been shown to be a global regulator of many exoprotein genes, and is the basis for the exemplary screen described herein. The screen looks for inhibitors of this kind of regulatory cascade. Other global regulators exist (Sar, Xpr, Sae, etc) that may contribute to expression patterns of these exoproteins and other genes.

FIGURE 2. Schematic of the P3- β -lactamase fusion. The basis for the exemplary screen is a fusion construct between the P3 promoter and a reporter gene (in this case, a β -lactamase). The production of RNAIII is critical in regulating downstream exoproteins. Fusion of the P3 promoter with an easily assayed reporter allows convenient monitoring of the effect of a given compound on RNAIII production.

FIGURE 3. The fusion construct is contained in the shuttle plasmid shown, which contains both an *E. coli* and an *S. aureus* origin of replication. The plasmid can be maintained with any of several selective agents, as it contains resistance determinants to erythromycin and chloramphenicol.

FIGURE 4. Graph of the temporal regulation and Agr dependence of the P3-driven β -lactamase activity. The plasmid shown in Figure 3 shows the expected phenotypes in Agr+ and Agr- host cells. Lacking key components of the Agr operon, the fusion is inert in the Agr- cells and little or no β -lactamase activity is detected. By contrast, as has been previously seen with RNAIII in Agr+ cells, the P3 promoter becomes active in mid-log phase, as evidenced by the sharp increase in β -lactamase activity.

FIGURE 5. Schematic example of the screen assay plates. Certain compounds of interest will affect the production of beta-lactamase without killing the cells. In the "growth control" plate, the fusion-containing Agr+ cells are tested with 80 different compounds in wells A2-H11. Columns 1 and 12 are devoted to controls as described in the text. After suitable exposure to compound, cell density is measured (O.D. 600 nm) and an aliquot is transferred to "P3- β -lactamase" assay plate for testing the activity of the reporter enzyme by a nitrocefin assay (O.D. 490 nm). Compounds are evaluated using the algorithms shown below. "Active" compounds may be acting to prevent P3 induction. Alternative hypotheses are explored in secondary assays.

25 SCREEN ALGORITHMS:

Growth in presence of test compounds:

$$[(T650-B650)+(C650-B650)] \times 100 =$$

% of growth in presence of test compound

- T650: Average growth of cells (8325-4/pMP25)
with test compound (O.D. at 650 nm)
- B650: Average media blank controls (O.D. at 650
nm)
- 5 C650: Average growth of control cells (8325-
4/pMP25) without compound (O.D. at 650 nm)

If score is <70%: Indicates a "growth inhibitor"
and compound is considered
"inactive".

- 10 If score is 70-80%: Indicates a "growth retardant
(GR)" and compound is considered
a potential "active" hit.

If score is ≥80%: Indicates a potential "active" hit.

Inhibition of β -lactamase activity:

- 15
$$\left(\left[\frac{(T490 - B490)}{(T650 - B650)} \right] + \left[\frac{(C490 - B490)}{(C650 - B650)} \right] \right) - 1 \times -100 = \% \text{ inhibition of } \beta\text{-lactamase activity due to}$$

test compound and corrected for cell density

- T650: Average growth of cells (8325-4/pMP25)
with test compound (O.D. at 650 nm)
- 20 B650: Average media blank controls (O.D. at 650
nm)
- C650: Average growth of control cells (8325-
4/pMP25) without compound (O.D. at 650 nm)
- T490: Average activity of β -lactamase in cells
exposed to test compound (O.D. at 490 nm)
- 25 B490: Average media and buffer blank controls

(O.D. at 490 nm)

C490: Average activity of β -lactamase in
untreated control cells (O.D. at 490 nm)

- 5 If score is $\geq 75\%$: Indicates an "active" compound (i.e.,
potential inhibitor of the Agr system
if not directly inhibiting the
reporter enzyme).
- If score is $< 75\%$: Indicates an "inactive" compound
(compound is rejected).
- 10 If score is $\leq -25\%$: Indicates an "Inducer" (i.e.,
potential inducer of the Agr system).

FIGURES 6-8. Chemical structures of confirmed screen
hits (Compounds 1-18) identified using the P3- β -
lactamase fusion screen.

- 15 FIGURES 9-12. Generic and sub-generic structures
describing Compounds 1-18 (screen hits) and related
compounds. Each generic structure is designated by a
number, and the corresponding sub-generic structure(s)
is designated by the same number followed by a letter.
- 20 The table below shows the correspondence between the
specific screening hits (Compounds 1-18) and the generic
and sub-generic structures.

Table 1		
Correspondence of Screening Hit Compounds, Generic Structures, and Sub-generic Structures		
Compound No.	Generic Structure No.	Sub-generic Structure No.
1	1	1A
2	2	2A
3		
4		2B
5	3	3A
6		
7	4	4A
8		
9	5	5A
10	6	6A
11	7	7A
12	8	8A
13	9	9A
14	10	10A
15	11	11A
16	12	12A
17	13	13A
18	14	14A

FIGURE 13. Dose-Response curve for three of the active compounds. Note that, even at concentrations that do

not appreciably alter growth, the compounds markedly reduce the P3-driven output of β -lactamase. It is also noteworthy that the dose response and growth characteristics are distinct for these three compounds.

- 5 FIGURE 14. Northern Blots for RNAlII and for RecA (control). A more direct assay of the effect of active compounds on the P3 promoter activity is performed on confirmed hits. Total RNA is isolated from treated and untreated cells when cultures reach similar density
- 10 (O.D. 600 nm). The RNA is probed on a Northern Blot with a RNAlII-specific probe (see text) or, as a control for total RNA, with a RecA-specific probe. Lane 1, Agr+. Lane 2, Agr-. Lanes 3-5, Agr+ treated with different active compounds. The Northern Blot shows
- 15 varying levels of reduction of RNAlII expression caused by the three active compounds. Glycerol monolaureate (GML) has no effect on RNAlII production (lane 6). Note that lane 7 shows a marked increase in RNAlII, because it contains RNA derived from cells treated with compound
- 20 6 that is considered a P3-inducer (as estimated by the increased activity of the β -lactamase reporter and the screen algorithms). The results shown here indicate the validity of the reporter system for monitoring up-regulation as well as down-regulation of the Agr operon.
- 25 FIGURE 15. Quantitation of Northern Blot from Figure 14. Probes were labeled in a manner that allowed chemiluminescent detection. This was captured on

phosphor screens and quantitated with a Molecular Imager (BioRad), or autoradiograms were photographed and quantitated using a program in the public domain .

FIGURE 16. Plate test for alpha-hemolysin.

- 5 Supernatants of bacterial cell cultures grown in the presence of some active compounds identified in the screen were spotted onto a rabbit blood-agar plate containing 1µg/ml ciprofloxacin to prevent bacterial grown on plates and allowed to incubate overnight at
- 10 35°C. Clearing is an indication of alpha-hemolysin activity, and opacity indicates inhibition or lack of hemolysin activity. Note that sub-MIC concentrations of active compounds inhibited alpha-hemolysin activity, while sub-MIC concentrations of traditional drugs like
- 15 clindamycin, ciprofloxacin, or vancomycin did not inhibit alpha-hemolysin activity. Supernatants from untreated Agr+ and Agr- cultures were also spotted as controls. NC: No cell culture control (cultivation media). Note that all supernatants were corrected for
- 20 cell density of individual cultures before spotting identical volumes on the plate.

- FIGURE 17. Immunodetection and quantitation of staphylococcal enterotoxin B (Seb) and alpha-hemolysin in treated and untreated cultures. Exoproducts from
- 25 treated and untreated cultures were assayed by dot blot using a specific anti-Seb or anti-alpha-hemolysin antibody and a second antibody that allowed chemiluminescent detection of immune complexes. The

chemiluminescence was captured on phosphor screens and quantitated with a Molecular Imager (BioRad).

FIGURE 18. Immunodetection of staphylococcal protein A in treated and untreated cultures. Cells were grown in presence of compounds 1, 2, or 3 to a similar density (O.D. 600 nm - 0.5-0.6). Cells were lysed using lysostaphin prior to separation of cell surface proteins by SDS-PAGE. After electrophoretic transfer of proteins from the gel onto a nitrocellulose membrane, detection of protein A on the blot was possible by using a specific anti-protein A antibody and a second antibody that allowed chemiluminescent detection of immune complexes. The chemiluminescence was captured by autoradiography.

FIGURE 19. Synergistic effect of compound 13 in combination with methicillin against a methicillin-resistant PBP 2a-producing strain of *S. aureus* (MRSA COL). Compounds were combined in a checkerboard assay performed in a microtiter plate in which a two dimensional matrix of the two compounds was created such that both compounds vary in concentration (see text). The minimal inhibitory concentration (MIC) of methicillin (the smallest concentration of methicillin that prevented visible growth of MRSA COL) was recorded for each test compound concentration.

FIGURE 20. Northern Blots for RNAIII detection in several clinical strains of *S. aureus* (no. 212, 213,

215, and 217) grown in the presence of Compound 10. Untreated strain 8325-4 (Agr+) is also represented and acts as a control to show the maximal amounts of RNAIII also detected in all clinical strains grown without the test compound. Total RNA was isolated from treated and untreated cells when cultures reach similar density (O.D. 600 nm). The RNA was probed on the Northern Blot with a RNAIII-specific probe or, as a control for total RNA, with a RecA-specific probe.

10 Description of the Preferred Embodiments

A. Pathogenesis Approach

 An alternative, or supplemental approach to traditional antibiotic therapy is to treat the pathogenesis, altering the conditions or processes which make the presence of the bacteria damaging to the host organism. The appropriateness of this approach is seen by recognizing that a large number of genes are essential for establishing an infection and for producing molecules which cause the damage to the host organism. A exemplary, clinically significant organism for which considerable information on pathogenesis-related gene products and the regulation of those products is available is *Staphylococcus aureus*.

Staphylococcus aureus synthesizes a large number of extracellular proteins that are important during pathogenesis. These include several cytolytic toxins (α -, β -, γ -, and δ - hemolysin), toxic shock syndrome toxin-1 (TSST-1), enterotoxins, leucocidin, the immunoglobulin binding protein A, coagulase which acti-

vates prothrombin, several hydrolytic enzymes, and others (Smith, The initiation of sporulation, pp. 185-210, In I. Smith et al. eds., Regulation of procaryotic development, American Society for Microbiology, Washington, D.C. (1989)). In *S. aureus*, as in most other bacteria, pathogenicity is multifactorial, and the genes that encode the virulence factors are often subject to coordinate regulation. These regulating systems respond to changes in the bacterial environment during the process of infection and adapt the expression of the virulence genes in an appropriate manner. In laboratory cultures, the majority of extracellular proteins from *S. aureus* are produced preferentially at the end of the exponential phase of growth (Abbas-Ali and Coleman, 1977, *J. Gen. Microbiol.* 99:277-282).

Several mutations with a pleiotropic effect on the production of this group of extracellular proteins have been described (Yoshikawa et al., 1974, *J. Bacteriol.* 119:117-122; Bjorklind and Arvidson, 1980, *FEMS Lett.* 7:203-206). One such mutation is a Tn551 insertion in a locus designated *agr* (Recsei et al., *Mol. Gen. Genet.* 202:58-61 (1986)), for Accessory Gene Regulator.

B. General Features of *agr*

Strains of *S. aureus* containing the above *agr* mutation show a decreased production of α -hemolysin, β -hemolysin, δ -hemolysin, TSST-1, enterotoxin B, epidermolytic toxins A and B, leucocidin, staphylokinase, nuclease, serine- and metallo-protease, and acid

phosphatase, whereas the production of certain other exoproteins including protein A and coagulase is increased as compared to the isogenic parental strain (O'Toole and Foster, 1986, *Microb. Pathog.* 1:583-594; 5 O'Toole and Foster, 1987, *J. Bacteriol.* 169:3910-3915; Recsei et al., *Mol. Gen. Genet.* 202:58-61 (1986); Gaskill et al., 1988, *J. Biol. Chem.* 263:6276-6280; Janzon et al., *Mol. Gen. Genet.* 219:480-485 (1989)). Thus, in general, agr⁺ strains show late log to post- 10 exponential induction of a set of secreted proteins, and repression of some surface proteins as compared to agr⁻ strains.

The Tn551 insertional mutation mentioned above has been localized to an open reading frame (ORF) 15 encoding a 241 amino acid polypeptide (Peng et al., *J. Bacteriol.* 170:4365-4372 (1988)). This ORF, which is called agrA, is part of a polycistronic mRNA of approximately 3 kb which contains three additional ORFs, agrB, agrC and agrD (Janzon et al., *Mol. Gen. Genet.* 20 219:480-485 (1989); Novick et al., *EMBO J.* 12:3967-3975 (1993); Novick et al., *Mol. Gen. Genet.* 248:446-458 (1995)), which are also involved in the regulation of exoprotein synthesis.

Thus, the agr system provides a global 25 regulator of pathogenesis (virulence) factors. As indicated above, agr encodes part of a regulatory cascade that controls expression of a large number of toxins and degradative enzymes that are produced during Staphylococcal pathogenesis (Peng et al., *J. Bacteriol.* 30 170:4365-4372 (1988); Janzon and Arvidson, *EMBO J.*

9:1391-1399 (1990); Novick et al., *Mol. Gen. Genet.*
248:446-458 (1995)).

The centerpiece of this regulation is a two-component regulatory system involved in signal transduction. AgrA and AgrC comprise a two-component regulatory system with features common to many bacteria (Parkinson and Kofoed, *Ann. Rev. Genet.* 26:71-112 (1992); Stock et al., *Micro. Revs.* 53:450-490 (1989)) and which show cross-species homology (Vandenesch et al., *FEMS Lett.* 111:115-122 (1993); Novick et al., *Mol. Gen. Genet.* 248:446-458 (1995)). Such systems contain a histidine kinase sensor/transmitter protein capable of sensing an environmental signal, autophosphorylation, and phosphotransfer, in addition to a response regulator protein that not only becomes phosphorylated by the histidine kinase, but is also instrumental in directly or indirectly regulating specific gene expression as a result of this phosphorylation. In their 1992 review, Parkinson and Kofoed cite more than 50 response regulators and more than 30 sensor/transmitters from at least 35 different bacteria (Parkinson and Kofoed, *Annu. Rev. Genet.* 26:71-112 (1992)). Most of these, including the Agr transmitter and receiver (at that time called AgrORF2 and AgrA), contain a canonical set of conserved residues and are termed orthodox transmitters or receivers. A notable exception is the AlgR2 gene product, involved in alginate regulation in *Pseudomonas aeruginosa*, which lacks or does not preserve certain sections conserved in the orthodox set.

The AgrA and AgrC proteins are the putative

signal transduction proteins that allow induction of the Agr P2 and P3 promoters in response to the bacterial environment (Kornblum et al., Agr: a polycistronic locus regulating exoprotein synthesis in *Staphylococcus aureus*, pp. 373-402, In R.P. Novick (ed.), Molecular Biology of the Staphylococci, VCH Publishers, Inc., New York (1990)). Transcription of the agr operon is activated at the end of the exponential growth phase by an agrAC-dependent mechanism (Peng et al., *J. Bacteriol.* 170:4365-4372 (1988); Novick et al., *Mol. Gen. Genet.* 248:446-458 (1995)). Cloned agrA was shown to complement the pleiotropic exoprotein defect seen with such mutations as Tn551 (Novick et al., *EMBO J.* 12:3967-3975 (1993)) which are located within agrA, but not phenotypically similar mutations located elsewhere in the agr locus. In more detail, recent publications (Guangyong et al. *Proc. Natl. Acad. Sci. USA.* 92:12055-12059 (1995); Balaban and Novick, *Proc. Natl. Acad. Sci. USA.* 92:1619-1623 (1995)) showed that a peptidic factor produced by *S. aureus* accumulates during growth and is responsible for activating the Agr response. This autocrine regulation of toxin production by *S. aureus* is mediated by an octapeptide derived by AgrB and AgrD. AgrC serves as the cell surface receptor for the AgrBD-derived octapeptide and subsequently activates AgrA by signal transduction. These studies indicate that *S. aureus* virulence is regulated by a density-sensing system which is homologous to other bacterial regulatory system autoinduced by homoserine lactones. Furthermore, the organization of the *S. aureus* system resembles the

competence-inducing *comAP* operon of *Bacillus subtilis* that also utilizes a peptide for autoinduction (Guangyong et al., *Proc. Natl. Acad. Sci. USA*, 92:12055-12059 (1995); Balaban and Novick, *Proc. Natl. Acad. Sci. USA*, 92:1619-1623 (1995)).

Activation of *AgrA* also leads to an increased transcription of the δ -hemolysin gene (*hld*) which is located immediately upstream of the *agr* operon encoding *AgrA* (Janzon et al., *Mol. Gen. Genet.* 219:480-485 (1989)) and which is transcribed from the divergent promoter, P3. Transcription from P3 produces a 0.5 kb transcript, RNAIII, which codes for δ -hemolysin. δ -Hemolysin is a small polypeptide of only 26 amino acids (Fitton et al., 1980, *FEBS Lett.* 115:209-212) which is secreted without a signal peptide (Fitton et al., 1980). An insertion in the RNAIII region, distal to the δ -hemolysin coding region inactivates *agr* global function, but does not interfere with the activity of the two promoters, P2 and P3. Since the signal transduction elements in the P2 operon are not affected, this indicates that a product of the P3 region encodes a critical regulator of the *agr* response (Novick et al., *EMBO J.* 12:3967-3975 (1993)). (Note that P3 and the *hld*/RNAIII gene is considered herein to be part of the *agr* locus.)

However, strains which are defective in *agrA* or *agrC* of the P2 operon are *agr*⁻ and lack both P2 and P3 transcripts. Specifically, since *agrA* is required for P3 transcription, and since P3 transcription is needed for *agr* activation of exoprotein synthesis, this

indicates that there are at least two sequential regulators. Induction of RNAIII under the control of a β -lactamase promoter in the absence of any other elements of the *agr* system activates both the positive and negative functions of the *agr* response, indicating that a P3 product is the actual regulator of the exoproteins. Mutation and deletion analysis indicate that RNAIII itself, rather than a translation product, is the effector. Further, the regulation by RNAIII is primarily at the transcription level, but in some cases also at translation. (Novick et al., *EMBO J.* 12:3967-3975 (1993).)

Thus, induction of RNAIII subsequently influences expression of many pathogenesis-related genes, and Figure 1 summarizes this global regulation (Janzon and Arvidson, *EMBO J.* 9:1391-1399 (1990); Novick et al., *EMBO J.* 12:3967-3975 (1993) Guangyong et al. *Proc. Natl. Acad. Sci. USA.* 92:12055-12059 (1995); Balaban and Novick. *Proc. Natl. Acad. Sci. USA.* 92:1619-1623 (1995); Novick et al., *Mol. Gen. Genet.* 248:446-458 (1995)).

In addition to the *agr* system, Figure 1 also identifies other gene products (Xpr, Sar, and Sae) from other global regulatory loci that were shown to interact with, influence, or contribute to the overall regulation of pathogenesis factors, including those shown to be regulated by the *agr* locus. For example, the regulatory locus *xpr*, identified by a Tn551 insertional inactivation of a chromosomal site distinct from the *agr* locus, also reduces the expression of several *S. aureus*

exoproteins. These observations suggest that *xpr* and *agr* behave as interactive global regulators (Smeltzer et al., 1993, *Infect. Immun.* 61:919-925).

Similarly, other studies have shown that
5 inactivation of the *sar* locus, also distinct from the *agr* and *xpr* loci, results in abnormal exoprotein expression (Cheung & Projan, 1994, *J. Bacteriol.* 176:4168-4172; Cheung et al., 1992, *Proc. Natl. Acad. Sci. USA* 89:6462-6466. Phenotypic and transcriptional
10 studies revealed that the *sarA* gene is required for the maximal expression of RNAIII and consequently shows an interactive regulation of exoproteins by both the *agr* and *sar* loci. Most recently, it was strongly suggested that the *sar* locus regulates binding to the P2 promoter
15 of the *agr* locus (Heinrichs et al., 1996, *J. Bacteriol.* 178:418-423). Such interactive regulation between the *sar* and *agr* loci should allow the identification of inhibitors of *sar* by the design of promoter-fusion screens. For example, for such a purpose, the P3
20 promoter of the promoter-fusion construct disclosed herein can be substituted by the P2 region to evaluate the inhibitory effect of test compounds on *sar* by measuring the level of expression of the reporter gene under the control of P2.

25 Another distinct regulatory locus, termed *sae*, was also identified by an insertional inactivation that produced a mutant defective in the production of several exoproteins (Giraud et al., 1994, *Can. J. Microbiol.* 40:677-681). However, in this case, the phenotypic
30 characteristics of the mutant were different from the

other insertional pleiotropic mutations, *agr*, *sar*, and *xpr*, and it is not known at this time exactly how this locus exerts its global regulation.

Finally, another locus, as yet unnamed, was
5 also shown to have pleiotropic effects on the expression of both exoproteins and cell wall associated proteins. The latter locus is also distinct from *agr*, *xpr*, and *sar*, but remains to be further investigated (Cheung et al., 1995, *J. Bacteriol.* 177:3220-3226).

10 Previous work by several laboratories has established that mutation of *agr* reduces virulence in mice. Smeltzer et al., *Infect. Immun.* 61:919-925 (1993) created Tn551 insertions at either the *agr* or the related *xpr* locus. No mortality was observed after
15 introduction of high inocula of either of these mutants to the peritoneal cavity of BalbC mice, as compared with 67-100% mortality of control mice injected with parent wild type strains.

In a mouse arthritis model, Abdelnour et al.,
20 *Infection and Immunity* 61:3879-3885 (1993) showed higher virulence of parent wild type strains than of *agr* or *hld* mutants (also created by Tn551 insertion), as measured by swelling, erythema, synovitis, and erosivity, as well as by histopathology and bacteriology. Interestingly,
25 bacteria were not culturable from joints of mice previously intravenously injected with either *agr* or *hld* mutants, as compared to the joints of mice intravenously injected with wild type *Staphylococci*, where 73% produced positive cultures. These experiments were
30 performed 21 days after intravenous injection.

A transcriptional fusion between a regulated promoter of the *agr* locus (P3) and a reporter gene (β -lactamase) was developed that provides a first-round screen for compounds that prevent Agr-mediated regulation (Figure 2). (A P3- β -lactamase fusion is described in Novick et al., 1995, *Mol. Gen. Genet.* 248:446-458.) The screen is based on a simple enzyme assay that can be performed on whole cell cultures grown with and without addition of the potential antipathogenic. To be classed as an antipathogenic, the compound should reduce activity of the reporter gene in the Agr+ background at sub-MIC (Minimum Inhibitory Concentration) levels. While the construction of a hybrid DNA including a promoter, P3, from a bacterial global pathogenesis regulator gene of the *agr* locus, the *S. aureus* RNAIII gene, linked to a reporter gene is described below, it should be recognized that the promoter from any global regulator of pathogens may be used. A reporter gene for use in the hybrid DNA construct can be any gene whose product is readily specifically detected, such as an enzyme which can produce a readily detectable effect. This specifically includes a β -lactamase gene, since a β -lactamase can be readily detected by colorimetric assay or by resistance to the presence of a cleavable β -lactam, like penicillin. However, the reporter could also be a different enzyme detectable by biochemical assay or a different resistance factor detectable by appropriate antibiotic selection.

This strategy has at least three significant virtues. First, the process can be repeated on virtual-

ly any promoter region that is identified as part of a global regulatory cascade, independent of additional (and time consuming) molecular and biochemical analysis of the gene product. Second, the screening system can be standardized for the reporter assay - inhibitors of different genes can be screened by an identical method. Third, such fusion assays can detect any upstream contributor to expression of the global regulatory pathway. Thus, inhibitors of several targets can be simultaneously sought.

As mentioned previously, the centerpiece of the regulation of pathogenesis by Agr is a two-component regulatory system involved in signal transduction. Because these signal transduction components (Novick et al., Mol. Gen. Genet. 248:446-458 (1995)), as well as the RNAIII transcript have cross-species homology (Vandenesch et al., FEMS Lett. 111:115-122 (1993); Axelsson et al., Appl. Environ. Microbiol. 59:2868-2875 (1993)), the effect of test compounds on the expression of virulence factors from other bacterial species is also investigated. Vandenesch et al. showed that sequences related to the *S. aureus agr* are present in *Staphylococcus lugdunensis*, using Southern blot analysis and sequencing data. The -10 and -35 elements of promoters P2 and P3 were highly conserved between the two species, as were several sequences at the putative transcription site for the *agr* promoters. In addition, the predicted amino acid sequences of *S. lugdunensis* *agr*-like gene products indicate a substantial degree of sequence similarity between the corresponding gene

products for the two species. Such information suggests a high probability that homologs of this global regulator (*agr*) will also be found in other species. Although possibly weak, the intrinsic growth inhibitory
5 property of test compounds on a large selection of bacterial species is also studied to identify other species whose two-component regulatory systems may be affected by test compounds.

Analogous screens can be produced using known
10 or novel global regulators or two-component regulatory systems. In this regard, it is notable that some of the confirmed hits from the Agr P3- β -lactamase fusion screen were shown to modulate either chemotaxis or sporulation (see below), processes which are also mediated by two-
15 component regulatory systems. Thus, such systems can be assayed microbiologically or biochemically, either secondarily to this or other assays, or novel primary screens can be created in the manner of the Agr Screen. A critical overlap is that these two-component
20 regulatory systems all cause the induction of at least one promoter, so it will invariably be possible to create a reporter-fusion, much as was done with the P3- β -lactamase fusion. Fusion assays are not uncommon, and at least one fusion assay for a two-component regulatory
25 system exists (Roychoudhury et al., PNAS 90:965-969 (1993)). In contrast to the narrow focus on the alginate system in Roychoudhury et al., this invention proposes a broad recognition of the utility of the Agr P3- β -lactamase screen for enriching in molecules that are
30 likely to be active on other orthodox two-component

regulatory systems, as opposed to atypical (unorthodox) systems such as the one described for alginate regulation (Parkinson & Kofoed, 1992, Ann. Rev. Genet. 26:71-112). Also, this invention recognizes the broad applicability of this kind of assay for identifying inhibitors of regulatory systems in particular (as opposed to other, more traditional targets such as molecules involved in cell wall or protein synthesis).

Thus, as shown below with some examples, inhibitors of two-component regulatory systems of more distant species controlling other important cellular functions can be found using the P3- β -lactamase fusion screen. In addition, by creating other fusion-specific screens, other regulatory systems can be more specifically exploited.

Examples of other specific regulatory systems, some of which are involved in the pathogenesis of particular organisms, are shown in Table 2.

20

25

Table 2			
Bacterial Two-Component Regulatory Systems			
System	Genes	Signal Transducer and Response Regulator	Organism
Staphylococcal virulence		AgrC/A (AgrORF2/A)* +	<i>Staphylococcus aureus</i> †
Staphylococcal virulence	<i>Xpr</i>	?	<i>S. aureus</i>
Staphylococcal virulence	<i>Sar</i>	?	<i>S. aureus</i>
Staphylococcal virulence	<i>Sae</i>	?	<i>S. aureus</i>
Staphylococcal virulence	<i>Agr-sl</i>	?	<i>Staphylococcus lugdunensis</i>
Xanthomonas virulence	<i>Xcc</i>	<i>Xcc2/Xcc1</i>	<i>Xanthomonas campestris</i> †

	Xanthomonas virulence		RpfC	<i>Xanthomonas campestris</i> †
	Bordetella virulence		BvgS/BvgA	<i>Bordetella pertussis</i> †
	Salmonella virulence	Vir	PhoQ/P	<i>Salmonella typhimurium</i> * †
	Agrobacterium virulence	Vir	VirA or VirA/G	<i>Agrobacterium tumefaciens</i> *
5	Pseudomonas virulence	Lem	LemA/GacA	<i>Pseudomonas syringae</i> , <i>P. viridiflava</i> , <i>P. fluorescens</i> †
	Pseudomonas virulence	Rep	RepA/?	<i>P. viridiflava</i> *
	Klebsiella virulence		?/MrkE	<i>Klebsiella pneumoniae</i> †
	Antibiotic Resistance	Van	VanR/S	<i>Enterococcus faecium</i> †
	Antibiotic Resistance	Bla	BlaR1/I	<i>S. aureus</i> *
10	Antibiotic Resistance	Mec	MecR1/I	<i>S. aureus</i> *
	Antibiotic Stress	RteAgr	RteA	<i>Bacteroides thetaiotaomicron</i> †
	Heavy Metal Stress	Cut	CutS/R	<i>Streptococcus lividans</i> †
	Heavy Metal Stress	Pco	?/PcoR	<i>Escherichia coli</i> †
	Enzyme secretion	Deg	DegS/U	<i>Bacillus subtilis</i> * †
15	Chemotaxis	Che	CheA/B or CheA/W	<i>E. coli</i> , <i>Enterobacter aerogenes</i> , <i>S. typhimurium</i> * †
	Nitrogen regulation	Ntr	NR11/I NtrB/A	<i>B. pertussis</i> , <i>E. coli</i> , <i>K. pneumoniae</i> , <i>K. aerogenes</i> , <i>S. typhimurium</i> *†
	Phosphate regulation	Pho	PhoR/B	<i>E. coli</i> , <i>B. subtilis</i> * †
	Phosphate regulation	Cre	CreC/B	<i>E. coli</i> †
	Oxygen regulation	Arc	CpxA/ArcA	<i>E. coli</i> * †
20	Porin expression osmolarity	Omp	EnvZ/OmpR	<i>E. coli</i> , <i>S. typhimurium</i> *†
	Sporulation	Spo	KinA/B (SpoIIJ)	<i>B. subtilis</i> * †
	Fruiting body formation	Frz	FrzE	<i>Myxococcus xanthus</i> *†
	Motility			
	Starvation	Asg	AsgA	<i>M. xanthus</i> †
25	Heptose phosphate uptake	Uhp	UhpB/A	<i>E. coli</i> * †
	Dicarboxylate transport	Dct	DctB/D	<i>Rhizobium leguminosarum</i> *
	Tricarboxylate transport	Tct	?/TctD	<i>S. typhimurium</i> *
	Phosphoglycerate transport	Pgt	PgtB/A	<i>S. typhimurium</i> *
	Redox	Nar	NarX/X/L	<i>E. coli</i> *
30	Competence	Com	?/ComA	<i>B. subtilis</i> *†
	Hydrogenase	Hyd	HydH/G	<i>E. coli</i> , <i>S. typhimurium</i> *

*As cited in Stock, J. S., A. J. Ninfa, A. M. Stock, 1989. Microbiol. Rev. 53:450-490.

† As cited in Parkinson, J. S., and E. C. Kofoid, 1992. Annu. Rev. Genet. 26:71-112.

‡ As cited in Ji, G., R. C. Beavis, and R. P. Novick, 1995. Proc. Natl. Acad. Sci. USA. 92:12055-12059.

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C. Description of primary screen

1. Fusion construction.

The Agr assay is designed to identify compounds which inhibit activation of the Agr P3 promoter, and
10 thereby reduce bacterial virulence. The experimental bacterial strain for the protocol is *Staphylococcus aureus* strain 8325-4/pMP25 (strain ISP479C/pMP25, Smeltzer et al, 1993, *Inf. & Imm.* 61:919-925) carrying a recombinant fusion of the Agr P3 promoter to a staphylo-
15 cocal β -lactamase gene (Figure 3). It also contains a resistance marker for erythromycin.

2. Rationale and steps involved in the construction of the P3: β -lactamase fusion.

A staphylococcal reporter gene (the *BlaZ* β -lactamase) was first placed in a shuttle vector for *E. coli* and *S. aureus*. This was done leaving a restriction site upstream of the reporter for inserting a promoter, in this case, P3 (P3 is synonymous with the *hld* promoter). In addition, if needed, this construct
25 allows the entire assembly to be removed from the shuttle vector for cloning into an integrative vector for eventual homologous recombination into genomic DNA by cutting with the outermost restriction enzymes flanking the fusion (P3: β -lactamase).

30 3. Components.

a. The shuttle vector pMIN164 was obtained

from the University of Minnesota (Greg Bohach). This vector is a fusion of the *E. coli* pBR328 cloning vector (GenBank Accession #L08858) and *S. aureus* plasmid pE194 (Horinouchi and Weisblum, J. Bacteriol. 150:804-814 (1982)), joined at the *Clai* site.

b. The β -lactamase gene was from *S. aureus* strain 76. This strain is a clinical isolate provided by Henry F. Chambers (San Francisco General Hospital) and was shown to produce a β -lactamase by a nitrocefin test (Becton Dickinson Microbiological Systems, Cokeysville, MD).

c. The *hld* promoter, P3, was retrieved from pEX07 obtained from S. Arvidson (Karolinska Institutet, Stockholm, Sweden). The pEX07 plasmid contains a 2149 bp *Bgl*III-*Pst*I fragment of the *agr* locus cloned into the cloning vector pSP64 (Janzon and Arvidson, *EMBO J.* 9:1391-1399 (1990)).

4. Method.

a. The endogenous *E. coli* ampicillin-resistance (Ap^R) gene from pMIN164 was removed as follows: pMIN164 was cut with restriction enzymes *Aat*II and *Bsa*I, removing an 853 bp fragment which includes most of the Ap^R gene. The ends were filled with T4 DNA polymerase making them blunt before the vector fragment was purified on Nusieve agarose and self-ligated.

b. The β -lactamase gene was obtained by PCR from *S. aureus* strain 76. Primers were chosen from the sequence of the published staphylococcal gene, *blaZ* (Wang and Novick, *J. Bacteriol.* 169:1763-1766 (1987)). Primers were also designed to contain a *Bam*HI site at the 5' end and a *Sal*I site at the 3' end for cloning into those sites within the tetracycline resistance marker in the pBR328 portion of the shuttle vector.

The primers used are described below. The restriction sites are shown in bold and the nucleotide positions, nt, refer to the sequence of *blaZ* published by Wang and Novick (*J. Bacteriol.* 169:1763-1766 (1987)).

A. Oligo "blaZ-1": 5' primer (SEQ ID NO. 1).
5'-CGGGATCCATAAAAATTACAAC TG-3' (24-mer)
BamHI nt 105 (*blaZ*)

B. Oligo "blaZ-2": 3' primer (SEQ ID NO. 2).
5'-ACGCGTCGACGAATATTAAAATTCCTTCATTAC-3'
(33-mer)

SalI nt 968 (*blaZ*)

Oligonucleotides were synthesized using the ABI Model DNA Synthesizer. PCR amplification was performed using DNA released from whole *S. aureus* strain 76 cells as the DNA template. Taq Polymerase from Perkins-Elmer was the source of enzyme. The

PCR product including restriction sites was 900 bp. The PCR product was digested with *Bam*HI and *Sal*I for cloning into the corresponding sites of pMIN164 having a deleted Ap^R (see step 1 above).

5 c. The *hld* gene promoter (P3) was obtained by PCR from pEX07. Primers were also designed to contain *Bam*HI sites at both 5' and 3' ends for joining to the cloned staphylococcal *bla*Z in the *Bam*HI site of the shuttle vector (see step 2 above).

10 The primers used are described below. The restriction sites are shown in bold and the nucleotide positions, nt, refer to the sequence of *hld* published by Janzon et al., *Mol. Gen. Genet.* 219:480-485 (1989).

15 A. Oligo "hld-1": 5' primer (SEQ ID NO. 3).
 5'-CGGGATCCTTTGTATTTAATATTTTAAC-3' (28-mer)
 BamHI nt 1016 (*hld*)

 B. Oligo "hld-2": 3' primer (SEQ ID NO. 4).
 5'-CGGGATCCGATCTAGTTATATTAAC-3' (27-mer)
20 **Bam**HI nt 925 (*hld*)

 Oligonucleotides were synthesized using the ABI Model DNA Synthesizer. PCR amplification was performed using plasmid pEX07 as the DNA template. Taq Polymerase from Perkin-Elmer was the source of
25 enzyme. The PCR product including restriction sites

was 117 bp. The PCR product was digested with *Bam*HI and ligated to the cloned staphylococcal *bla*Z in the *Bam*HI site of the shuttle vector.

d. The proper configuration of the fusion
5 construct was verified by restriction digests, PCR
amplifications and sequencing. Sequencing was
performed using the Sequenase protocol (version 2.0,
USB, Cleveland, OH) and the hld-1 and hld-2 primers.
The resulting map of the recombinant plasmid pMP25
10 is illustrated in Figure 3.

e. The recombinant plasmid pMP25 isolated
from *E. coli* was placed into *S. aureus* by
electroporation of strain RN4220, a restriction
deficient derivative of strain 8325-4 used as
15 primary recipient for plasmids propagated in *E. coli*
(Janzon and Arvidson, *EMBO J.* 9:1391-1399 (1990)).
Thereafter, transduction experiments using
bacteriophage ϕ -11 allowed transfer of pMP25 from *S.*
aureus RN4220 to both strain 8325-4 (ISP479C) (an
20 *Agr*+ background) and strain RN6911. Strain RN6911
is an *Agr* null mutant (*Agr*-) in which a 3359 bp
fragment of the genomic *agr* locus was replaced with
a 3000 bp fragment encoding tetracycline resistance
(Nesin et al., *Antimicrob. Agents Chemother.*
25 34:2273-2276 (1990); Novick et al., *EMBO J.*
12:3967-3975 (1993)). Tests using *S. aureus* strains
8325-4/pMP25 (ISP479C/pMP25) and RN6911/pMP25 were
then performed to confirm the proper regulation of

β -lactamase (BlaZ) expression under the control of the P3 promoter (see below).

Although copy number and stability of the plasmid did not represent problems in preliminary
5 screening, the fusion construct can also be integrated in the chromosome if such problems occur in specific circumstances. The negative control strain for this assay is *S. aureus* RN6911/pMP25 (an *agr* null mutant, Agr-). Neither strain expresses detectable (endogenous)
10 β -lactamase in the absence of plasmid.

Staphylococcal exotoxins are produced in the early stationary phase of growth which mimics *in vivo* growth. *In vitro* expression of β -lactamase from the P3: β -lactamase fusion construct showed appropriate
15 temporal regulation (induction in the early stationary phase of growth) as shown in Figure 4. Also, the Agr dependence of expression of β -lactamase from the P3- β -lactamase fusion was demonstrated by comparing expression in Agr+ and Agr- backgrounds (Figure 4).

20 5. Compounds to be screened

The screening method of the present invention is appropriate and useful for testing compounds from a variety of sources for possible inhibitor activity. The initial screens were performed using a diverse library
25 of compounds, but the method is suitable for a variety of other compounds and compound libraries. Such compound libraries can be combinatorial libraries, natural product libraries, or other small molecule

libraries. In addition, compounds from commercial sources can be tested, as well as commercially available analogs of identified inhibitors.

Further, the methods are suitable as part of a medicinal chemistry program to identify derivatives and analogs of screening hits also having activity on global regulators of pathogenesis genes. Typically, such a program is directed to finding compounds having greater activity and/or other improved pharmacologic characteristics (e.g., improved characteristics relating to solubility, toxicity, and stability). Thus, such derivative and analogs can be evaluated or screened for the appropriate activity and other characteristics determined. For example, the methods of screening described herein can be used to determine the activity of such compounds. In particular, the compounds described herein as screening hits, compounds having structures corresponding to the described generic structures, and other derivatives of those compounds can be evaluated or screened, and can themselves provide the bases for further derivatization and screening. As indicated above, some analogs can be obtained from commercial sources; others can be obtained by chemical modification of available compounds or synthesized by methods known to those skilled in the art.

Since many of the compounds in libraries such as combinatorial and natural products libraries, as well as in natural products preparations, are not characterized, the screening methods of this invention provide novel compounds which are active as inhibitors

or inducers in the particular screens, in addition to identifying known compounds which are active in the screens. Therefore, this invention includes such novel compounds, as well as the use of both novel and known compounds in pharmaceutical compositions and methods of treating.

Example 1: Initial Screen. The Agr screen is performed as follows. Agr+ cells harboring the plasmid with the P3: β -lactamase fusion are diluted from a fresh overnight culture in Trypticase Soy Broth (TSB) to an optical density at 600 nm (O.D. 600 nm) between 0.01 and 0.015. As a positive control, four wells of a 96-well microtiter dish contain these dilute Agr+/ P3: β -lactamase cells without added compound. As a negative control for Agr P3 activity, four wells contain Agr-cells harboring the same plasmid, grown and diluted in the same manner as the Agr+ cells. As a negative control for growth, eight wells contain TSB with no inoculum. To test the activity of compounds, 180 μ l of the dilute Agr+/ P3: β -lactamase inoculum are placed in the remaining wells, which contain 20 μ l test compounds at 100 μ g/ml (10 μ g/ml final) and the plate is placed in a humidified incubator at 35°C for 6 hours. At this point the O.D.600 for the plate is read, and 20 μ l from each well are transferred to a fresh 96-well plate containing 80 μ l/well 0.25mM nitrocefin (a chromogenic cephalosporin) which becomes red upon hydrolysis by β -lactamase enzymes with a maximal absorbency at 490 nm. A compound is considered a hit when β -lactamase activity

is significantly inhibited, and growth is not significantly inhibited by that test compound at the same concentration. Figure 5 shows an example of a screen plate containing a hit (well A4 has no β -lactamase activity although growth was unaffected). For data analysis, the algorithms shown in the brief description of Figure 5 are used to calculate results. Based on those results the test compounds can be characterized according to biological activity as, for example, a growth inhibitor, a growth retardant, an inactive compound (no effect), an active compound (a potential inhibitor of the Agr system), or an inducer (potential inducer of the Agr system).

D. Secondary Evaluation

Raw hits detected in the primary Agr screen may be β -lactamase inhibitors, weak or selective protein synthesis inhibitors, environmental signal inhibitors, broad signal transduction inhibitors, specific AgrAC inhibitors, or inhibitors acting on regulation steps other than AgrAB, possibly Xpr, Sae and Sar (Smeltzer et al., *Infect. Immun.* 61:919-925 (1993); Cheung and Projan, *J. Bacteriol.* 176:4168-4172 (1994); Giraudo et al., *Can. J. Microbiol.* 40:677-681 (1994); Heinrichs et al. *J. Bacteriol.* 178:418-423 (1996)) and others (Cheung et al. *J. Bacteriol.* 177:3220-3226 (1995)). Therefore, the effect of hits on non functional mutants of such genes (*xpr*, *sae*, and *sar*) or of individual components of regulatory operons (ex. either AgrA, B, C, or D in the Agr operon), as well as a battery of secondary tests

are appropriate to better characterize screen hits and to elucidate their mode of action. Secondary characterization of hits may be done using the tests outlined below.

5 Example 2: β -Lactamase inhibition studies.

β -Lactamase inhibition studies are performed with isolated Staphylococcal enzyme purified from *S. aureus* strain 76 and from which the β -lactamase gene was used in the fusion construct. β -lactamase purification was
10 done by the method of Kernodle et al., *Antimicrob. Agents Chemother.* 34:2177-2183 (1990) using a cellulose phosphate cation exchange matrix. The isolated enzyme is pre-incubated with various concentration of test
15 conditions) prior to the addition of the chromogenic substrate nitrocefin to follow β -lactamase activity (O'Callaghan et al., *Antimicrob. Agents Chemother.* 1:283-288 (1972)). Clavulanic acid is used as the inhibitor control. To be carried forward for further
20 characterization and evaluation, a hit should not appreciably inhibit β -lactamase (or other reporter enzyme being used).

Example 3: RNAIII and exotoxins. An Agr inhibitor should alter expression of RNAIII and of the Agr-regu-
25 lated exotoxins. Expression of hemolysins α , β and δ , and of DNase, enterotoxin B, protease V8 and lipase is normally positively controlled by Agr due to the activation of the P3 promoter of RNAIII. An Agr

inhibitor should decrease expression of some or all of these proteins and of the RNAIII transcript and indicator tests can be performed as follow:

RNAIII transcription. RNAIII levels are measured by Northern Blot analysis using a modification of the method of Janzon and Arvidson, *EMBO J.* 9:1391-1399 (1990). Briefly, a 368bp probe was created by PCR using primers designed to amplify the RNAIII fragment between base pairs 1201 and 1569 of the map obtainable by GenBank accession number. (RNAIII itself is encoded between base pairs -1050-1570.) The template used for PCR was pEX07 (Janzon and Arvidson, *EMBO J.* 9:1391-1399 (1990)). The probe is labeled with UTP-fluorescein using a commercially available kit. Agr- and Agr+ (with and without inhibitors) are cultured in the presence of a maintenance amount of erythromycin (with putative inhibitors, as appropriate) and RNA extracts are performed at various time points (or at specific O.D. levels). The purified total RNA is run on formaldehyde-agarose gels and transferred overnight to charged nylon. The nylon blot is then hybridized overnight with labeled probe and signal is detected using commercially available anti-fluorescein-HRP conjugated-antibody and chemiluminescent reagents. Film is exposed to the freshly worked up blots and developed using an automated film processor. Alternatively, a chemiluminescence-sensitive screen can be used for exposure, and quantitation can be performed on a molecular imager.

In all RNAlII quantitation experiments, both ethidium bromide staining of the gel prior to transfer and the use of a second probe (a RecA gene probe) were used to verify that essentially equivalent amounts of total RNA were loaded in each lane. The RecA probe was constructed as follows: PCR primers were designed to amplify a 1.1 kb fragment from the RecA gene which sequence was obtainable by GenBank accession number L25893. The genomic DNA template was from *S. aureus* strain 8325-4 and the primers were:

recA 5'-CCCTATGTGATGTTTAGCTC-3'

recA 5'-TTAGGAGGTCTCGCTATGGA-3'

The RNAlII probe was labeled with UTP-fluorescein and detection was performed as described above.

The following tests (hemolysins, lipase, DNAase, enterotoxinB, and protease) are provided for further characterization of hits. Those hits that are effective inhibitors of the Agr response will lower the level (or perceptible activity) of one or more of these enzymes. Some hits may be general signal transduction inhibitors, with less effect on these specific enzymes, but still of interest; these will be identified through the next set of tests under Example 5.

α , γ , δ , and β -hemolysin activity and expression.

α -hemolysin and β -hemolysin activity are measured against rabbit or sheep red blood cells, respectively, by titration assay with 0.5-1% blood cells and/or by spot assay for clearing on blood agar plates (Peng et al., *J. Bacteriol.* 170:4365-4372 (1988)). α , γ , δ , and β -hemolysin

expression can also be measured by the methods of Cheung and Ying, *J. Bacteriol.* 176:580-585 (1994) and Janzon and Arvidson, *EMBO J.* 9:1391-1399 (1990), using standard immunoblot techniques. Briefly, a
5 small amount of concentrated extracellular fluid is spotted or electroblotted onto nitrocellulose membrane. The membranes are incubated with specific and appropriate anti-hemolysin antibodies.

Detection is via any of several available anti-anti-
10 body-enzyme conjugates and commercially available reagents (ex. Sigma Chemicals, Co.).

Lipase. Lipase production is measured on 1% Tween agar plates by measuring clearing around a predetermined quantity of sample (Cheung and Ying, *J. Bacteriol.* 176:580-585 (1994)). A more specific assay
15 for Tributyrin hydrolysis is performed using the method of Smeltzer et al., *Applied and Env. Microbiol.* 58:2815-2819 (1992). Lipase activity is assayed spectrophotometrically by the decrease in
20 absorbance at 450nm of suspensions of the triglyceride stabilized with low-melt or standard agarose. It has also been possible to adapt commercially available kits for measuring human lipase for the purpose of measuring bacterial lipase.

25 DNase. Culture supernatants are placed in wells in Toluidine Blue DNA (TBD) agar. DNase activity is assessed by the presence of a pink zone around the well (Shortle, *Gene* 22:181-189 (1983); Patel et al., *Infection and Immunity* 55:3103-3110 (1987)).

Enterotoxin B. Enterotoxin B can be detected by using standard immunoblot techniques with specific antibodies commercially available (ex. Sigma Chemicals, Co.).

- 5 Protease. Staphylococcal protease V8 activity can be detected by using the commercially available chromogenic substrate carbobenz-oxy-L-phenylalanyl-L-leucyl-L-A glutamic acid-4-nitroanilide (Boehringer Mannheim, Corp.).

- 10 Example 4: Protein A and Coagulase. Expression of protein A and of coagulase is normally negatively controlled by Agr, and an Agr inhibitor should increase expression of these proteins. Indicator tests are performed as follows:

- 15 Protein A. Protein A expression is assayed by immunoblots of SDS-Page separated total cell proteins (Patel et al., *Infection and Immunity* 55:3103-3110 (1987)).

- 20 Coagulase activity. Culture supernatants are tested for coagulase activity by incubation (37°C, 18 hrs.) with neat rabbit plasma (Patel et al., *Infection and Immunity* 55:3103-3110 (1987)).

- 25 Example 5: Signal transduction systems and Secretion. Compounds acting as general signal transduction inhibitors are of special interest, as such inhibitors may be novel antimicrobial agents. There are several well-characterized or putative bacterial

two-component signal transduction systems that can be targeted (see Table 2). Certain of these can easily be used to test the compounds for general signal transduction inhibition. These include but are not limited to: alkaline phosphatase induction, sporulation (*Bacillus subtilis*), and chemotaxis (*B. subtilis*, *Escherichia coli*, and *Salmonella typhimurium*). In addition, many resistance mechanisms are signal transduction mediated, for example vancomycin resistance, β -lactamase resistance, and methicillin resistance. Finally, signal transduction inhibitors can be distinguished from inhibitors of basic secretion. Agr hits that are not secretion inhibitors but which inhibit one or more of the following signal transduction pathways will be carried forward as general signal transduction hits.

Alkaline Phosphatase induction: Phosphate utilization has been extensively characterized in *E. coli*, and is now well-studied in *B. subtilis* as well. In both species, there is a level of regulation that occurs via a two-component regulatory system; *phoR/phoB* in *E. coli* (Nakata et al., Genetic and biochemical analysis of the phosphate specific transport system in *Escherichia coli*, pp. 150-155, In A. Torriani-Gorini et al. ed., Phosphate metabolism and cellular regulation in microorganisms, American Society for Microbiology, Washington, D.C. (1987); Wanner, Phosphate regulation of gene expression in *Escherichia coli*, pp. 1326-1333, In F.C. Neidhardt et

al. eds., *Escherichia coli* and *Salmonella typhimurium*: cellular and molecular biology, American Society for Microbiology, Washington, D.C. (1987)) and *phoR/phoP* in *B. subtilis* (Seki et al., *J. Bacteriol.* 169:2913-2916 (1987); Hulett et al., *J. Bacteriol.* 176:1348-1358 (1994)). In *S. aureus*, the phosphatase that is active at pH 8 has been shown to be constitutive in some species and repressed by phosphates in others (Soro et al., *J. Clin. Microbiol.* 228:2707-2710 (1990)), indicating the possible presence of a two-component regulatory system. Alkaline phosphatase is induced in all three organisms after growth in phosphate-limiting media, and can be easily and sensitively assayed using whole cells, supernatants, or various cell fractions. The sample is combined with a colorimetric substrate, p-nitrophenyl phosphate, in a suitable buffer (100mM Tris, pH 8 with 0.5mM MgCl₂). If alkaline phosphatase is present, the colorless p-nitrophenyl phosphate is cleaved and releases yellow p-nitroaniline. Therefore, enzyme activity can be monitored by the increase in yellow color using a spectrophotometer at 405-410nm. If a compound interferes with the signal transduction pathway for phosphatase regulation, then growth of the organism in low phosphate medium in the presence of the compound will yield one of two results: it will lower the output of phosphatase per cell as compared to the cultures grown in the absence of compound in low phosphate medium; or, if the phosphate recovery

system is sufficiently disabled, it will cause a large decrease in cell growth as compared both to growth in the absence of compound and in low phosphate medium, or in the presence of compound in high phosphate medium.

Sporulation: Sporulation is a well-characterized event in many organisms, including *Bacillus subtilis* (*spoIIJ/spo0A* or *spoIIJ/spo0F*, Losick et al., *Annu. Rev. Genet.* 20:625-669 (1986); Smith, The initiation of sporulation, pp. 185-210, In I. Smith et al. eds., *Regulation of procaryotic development*, American Society for Microbiology, Washington, D.C. (1989)). It can be induced in various media (Leighton and Doi, *J. Biol. Chem.* 246:3189-3195 (1971); Piggot and Curtis, *J. Bacteriol.* 169:1260-1266 (1987)), and can be easily assayed (Nicholson and Setlow, *Sporulation, germination, and outgrowth*, pp. 391-429, In C.R. Harwood and S.M. Cutting eds., *Molecular biological methods for Bacillus*, John Wiley and Sons, Ltd., Chichester, West Sussex, England (1990)). Briefly, by heating the cells to 80-85°C for 15-20min, undifferentiated cells are killed while spores survive. Therefore, by determining the difference in viable cell count of control cultures versus heat treated cultures, it is possible to calculate the percent sporulation for a given culture or condition. Compounds that inhibit the signal transduction system responsible for the induction of sporulation will lower the viable cell count of the heat treated culture.

Chemotaxis: Motility has been studied in a variety of bacteria. The molecular mechanisms of motility have been best characterized in *E. coli* and *S. typhimurium* but analogs of the molecular components have been

5 detected in both Gram-negative and Gram-positive organisms (Morgan et al., *J. Bacteriol.* 175:133-140 (1993)). A two-component regulatory system composed of either *cheA/cheY* or *cheA/cheB* (Stewart and Dahlquist, *Chem. Rev.* 87:997-1025 (1987); MacNab,

10 *Motility and Chemotaxis*, pp.732-759, In F.C. Neidhardt, et al., ed., *Escherichia coli* and *Salmonella typhimurium*: cellular and molecular biology, American Society for Microbiology, Washington, D.C. (1987)), is central to the ability

15 of these organisms to detect and respond appropriately to chemical substances. Such response can be detected on minimal swarm agar plates containing a chemoattractant (sugars or amino acids) (J.S. Parkinson, *J. Bacteriol.* 126:758-770 (1976)),

20 or a capillary assay system, in which chemoattractant is placed in a capillary tube and immersed in a liquid bacterial culture. Bacteria capable of chemotaxis will swim into the capillary and can be detected by performing viable cell counts (Adler, J.

25 *Gen. Microbiol.* 74:77-91 (1973)). Bacteria are to be tested with capillaries or swarms containing a known chemoattractant, with chemoattractant and test compound in the assay medium, and with no chemoattractant but with test compound in the assay medium

30 to determine the extent of suppression of chemotaxis

caused by a given test compound. Inhibitors of the signal transduction components involved in chemotaxis should modulate the ability of the bacteria to swim into the capillary tube, thereby changing the viable cell count, or alter the diameter of bacterial swarms.

Induction of antibiotic resistance. Vancomycin resistance in *Enterococci* is a complex process that is initiated through a signal transduction pair, *vanR/ vanS* (Arthur et al., *J. Bacteriol.* 174:2528-2591 (1992); Wright et al., *Biochemistry* 32:5057-5063 (1993)). Using inducible vancomycin resistant strains of *Enterococcus faecium*, it is possible to detect inhibitors of the initial step in resistance (induction of resistant genes in the presence of vancomycin) by performing synergy studies with vancomycin. An inhibitor of the signal transduction pair should cause such an organism to become more sensitive to vancomycin, reducing the MIC to vancomycin.

β -lactamase induction. Studies on the induction and secretion of β -lactamase as well as synergy studies with β -lactams in *S. aureus* strains carrying the plasmid p1258 will provide answers to several questions. Expression of the *blaZ* β -lactamase is normally induced by β -lactam molecules through the *blaR1/blaI* signal transduction pathway (Wang et al., *Nucl. Acids Res.* 19:4000 (1991); Bennett and Chopra, *Antimicrob. Agents Chemother.* 37:153-158 (1993)), and inhibitors of this non-traditional signal

transduction pathway should be detected by induction studies with cells pre-exposed to the test compounds. If inhibitors are found, it should also be possible to show that they are synergistic with traditional β -lactam drugs. In addition, it has recently been shown that the *blaR1/ blaI* system may engage in crosstalk with the signal transduction pair involved in staphylococcal methicillin resistance, *mecR1/ mecI*, which regulate the expression of the major methicillin resistance factor, PBP2a (Tesch et al., *Antimicrob. Agents Chemother.* 34:1703-1706 (1990); Hiramatsu et al., *FEBS Lett.* 298:133-136 (1992)). Therefore, synergy studies on methicillin-resistant *S. aureus* (MRSA) will also be of use in demonstrating general signal transduction inhibition.

Compounds that show inhibitory activity in these or other microbial signal transduction systems will be of interest and will be carried forward for further study, including in vivo study.

Secretion may be evaluated with the same system using, this time, cells already induced for β -lactamase production prior to exposition to test compounds. Those compounds that create defects in secretion of β -lactamase will be considered to be in a separate category from signal transduction inhibitors, unless the cause of the secretion defect is eventually shown to originate in signal transduction inhibition.

Example 6: Membrane interactions. To study the

possible non-specific effect of the Agr screen hits on systems that involve membrane protein function in *S. aureus*, the lactose metabolism pathway may be used (Rosey et al., *J. Bacteriol.* 173:5992-5998 (1991);
5 Simoni et al., *J. Biol. Chem.* 248:932-940 (1973)). Growth of *S. aureus* on lactose leads to the induction of the lactose phosphoenolpyruvate phosphotransferase system and the effect of test compounds (hits) on lactose utilization may be evaluated by monitoring
10 growth in a defined medium.

Also, cadmium resistance in *S. aureus* carrying plasmid p1258 is due to a specific active efflux pump system (Silver and Walderhaug, *Microb. Rev.* 56:195-228 (1992)) and the effect of test compounds on cadmium
15 resistance may be investigated to provide information on their effects on membrane protein functions.

Finally, fluorescent hydrophobic probes may also be used to study direct effects of test compounds on membrane integrity.

20 Example 7: Protein synthesis. Additional information on hits may be obtained by studying the effects of test compounds on protein synthesis using a traditional assay system.

E. Pharmacology and in vitro testing.

25 In addition to secondary assays for determination of the mode of action of the primary hits, other in vitro tests are also appropriate for collecting pharmacological data, such as the following:

Example 8: Synergism/Antagonism studies. Standard checkerboard assays are designed to examine drug interactions *in vitro*. Generally, a two dimensional matrix of two drugs (or test compound versus known drug) is created such that both drugs vary in concentration. The effect of the various combinations is measured by examining the MIC or MBC for the particular set of concentrations and comparing this to the summed effects of the drugs alone using a relation known as the fractional inhibitory concentration index (FIC index). In this screen, the effects of the drug combinations on the β -lactamase activity under the control of the P3 promoter as well as the antibacterial activity of the known drugs and combinations can be monitored. The FIC index will provide the type of drug interactions: additive, synergistic, or antagonistic (Eliopoulos and Moellering, *Antimicrobial Combinations*, p. 432-492, In Lorian (Ed.), *Antibiotics in Laboratory Medicine*, 3rd ed., The Williams & Wilkins Co., Baltimore (1991)). A non-standard index based on the FIC index can be developed using the concentration at which a preselected percent inhibition of β -lactamase activity is observed in place of the MIC, and this index will provide a measure of whether the drugs interact with respect to antipathogenesis activity.

Example 9: Spectrum of activity. The effect of test compounds on the expression of virulence factors from several clinical strains of *S. aureus* may be determined. Initial tests that can be performed on various strains

grown in the presence of the test compounds include detection of RNAIII, α -hemolysin, DNase and lipase by agar plate assays as well as a coagulase slide test and immunodetection tests for enterotoxin B and protein A.

5 Example 10: Extended spectrum of activity. The effect of test compounds on growth of other organisms may also be assessed using standard MIC testing (microdilution or agar dilution).

10 Example 11: Toxicity. Potential toxicity to eukaryotes is preferably tested *in vitro* using yeast or mammalian cell cultures and colorimetric tests for prediction of acute toxicity *in vivo* (Garle et al., *Toxic. in Vitro*, 8:1303-1312 (1994)).

15 Example 12: Serum effects. The effect of serum on the observed activity of the test compounds is also evaluated (i.e., serum protein binding, serum inactivation of compounds, etc.).

20 Example 13: Analog testing. Structural analogs of hits are purchased, synthesized, or otherwise obtained from various sources for testing in the Agr screen as an initial structure activity relationship study.

Such synthesis of analogs, or chemical modification of identified screening hits, generally utilize synthetic and chemical modification methods known to
25 those skilled in the art. In certain circumstances, analogs which are synthesized or created by chemical

modification from identified screening hits are novel compounds. Such novel compounds which are active in the screening methods of this invention and/or are active on global pathogenesis regulators are within the scope of
5 this invention.

F. Pharmacology and in vivo testing.

Example 14: In vivo evaluation of microbial virulence and pathogenicity. Confirmation of specificity of activity and other in vitro evaluations
10 is done before in vivo testing is begun. The criteria for evaluation in vivo includes ability of the bacteria to replicate, the ability to produce specific exoproducts involved in virulence of the organism, and the ability to cause symptoms of disease in the animals.
15 In vivo evaluation follow protocols developed in light of the specific activities of the Agr inhibitors. Efficacy of the inhibitors alone and in combination with antibiotics is studied. The ability to down-regulate specific staphylococcal virulence factors is examined.

20 Six exemplary animal infection models appropriate for use to evaluate the effects of Agr screen hits are described below. The animal models are selected for efficiency, reproducibility and cost containment. Rodents, especially mice and rats, are
25 generally the preferred species as experimentalists have the greatest experience with these species. Manipulations are more convenient and the amounts of materials which are required are relatively small due to

the size of the rodents.

The mouse soft tissue infection model is a sensitive and effective method for measurement of bacterial proliferation. In this model, anesthetized mice are infected
5 with the bacteria in the muscle of the hind thigh. The mice can be either chemically immune compromised (e.g. cyclophosphamide treated) or immunocompetent. The dose of microbe necessary to cause an infection is variable and depends on the individual microbe, but commonly is
10 on the order of 10^6 colony forming units per injection for bacteria. A variety of mouse strains are useful in this model although Swiss Webster and DBA2 lines are most commonly used. Once infected the animals are conscious and show no overt ill effects of the
15 infections for approximately 12-24 hours, depending on the strain used. After that time virulent strains cause swelling of the thigh muscle, and the animals can become bacteremic within approximately 24 hours. This model most effectively measures proliferation of the microbe,
20 and this proliferation is measured by sacrifice of the infected animal and counting viable cells from homogenized thighs.

The diffusion chamber model is useful as second model for assessing the virulence of microbes (Dargis et al.,
25 *Infect. Immun.* 60:4024-4031 (1992); Malouin et al, *Infect. Immun.* 58:1247-1253 (1990)). In this model, rodents have a diffusion chamber surgically placed subcutaneously or in the peritoneal cavity. The chamber can consist of a polypropylene cylinder with
30 semipermeable membranes covering the chamber ends.

Diffusion of interstitial or peritoneal fluid into and out of the chamber provides nutrients for the microbes. The progression of the "infection" can be followed by examining growth, the exoproduct production or RNA
5 messages. The time course experiments are done sampling multiple chambers. Bacterial toxins and other exoproducts are produced and are measurable from cells grown in these chambers. Bacteria can persist at high concentrations for up to at least several days in this
10 model.

The endocarditis model is an important animal model effective in assessing pathogenicity and virulence for bacteria. . Either a rat endocarditis model or a rabbit endocarditis model can be used to assess colonization,
15 virulence and proliferation of bacteria in animals treated with test compounds having antipathogenic activities.

The osteomyelitis model is a fourth model useful in the evaluation of pathogenesis. Rabbits are used for these
20 experiments. Anesthetized animals have a small segment of the tibia removed and microorganisms are microinjected into the wound. The excised bone segment is replace and the progression of the disease is monitored. Clinical signs, particularly inflammation
25 and swelling are monitored. Termination of the experiment allows histologic and pathologic examination of the infection site to complement the assessment procedure.

The murine septic arthritis model is a fifth model relevant to the study of microbial pathogenesis. In this
30

model mice are infected intravenously and pathogenic organisms are found to cause inflammation in distal limb joints. Monitoring of the inflammation and comparison of inflammation vs. inocula allows assessment of the virulence of bacteria in animals treated with test compounds having antipathogenic activities.

Bacterial peritonitis offers rapid and predictive data on the virulence of strains. Peritonitis in rodents, preferably mice, can provide essential data on the importance of targets. The end point may be lethality or clinical signs can be monitored. Variation in infection dose in comparison to outcome allows evaluation of the virulence of bacteria in animals treated with test compounds having antipathogenic activities.

G. Screen results.

Table 3 shows the characteristics of several of the hits identified in the screen, and how they were classified as active compounds (putative Agr inhibitors or inducers) by the screen algorithms described herein (see Brief Description of Figure 5). The compounds are defined as "active" (inhibitor) or "inducer" in the Agr P3- β -lactamase fusion assay. The percent growth compared to untreated Agr+ cells and the % inhibition of β -lactamase (after correction for cell density) is shown for various concentrations of several hits.

Using such screen design and calculations, traditional drugs such as ciprofloxacin or vancomycin do not behave as hits using these algorithms.

Table 3			
Confirmed Hits from the Agr P3- β -lactamase Fusion Assay with Results derived from the Screen Algorithm			
Compound	Growth (%)	β -Lactamase inhibition (%)	Status from algorithm
1	105	75	Active
2	122	84	Active
3	70	83	GR and Active
4	113	86	Active
5	84	-36	Inducer
6	72	-111	GR and Inducer
7	103	87	Active
8	101	84	Active
9	91	86	Active
10	107	84	Active
11	104	77	Active
12	124	76	Active
13	111	75	Active
14	94	77	Active
15	101	76	Active
16	106	78	Active
17	87	83	Active
18	89	75	Active

Figures 6-8 provide the chemical structures of 18 hit compounds found in the screen. The structures of the specific hits shown in Figs. 6-8 are also represented by generic structures (Fig. 9-12), which correspond to the broader classes of compounds likely to have similar activity.

Titration assays were used to characterize hits and demonstrated a dose response of β -lactamase

expression with rising test compound concentration in several cases (Figure 13), indicating a specificity in the mode of action of such hits.

Subsequent characterization studies on many of the hits has also shown that these hits were not specific inhibitors of the Staphylococcal β -lactamase enzyme used as the reporter molecule in the screen (data not shown).

In addition to the active hits characterized as putative Agr inhibitors, two molecules were identified (Compounds 5 and 6, Table 3) that enhance the production of β -lactamase, indicating possible induction of the P3 promoter. These molecules will be referred to as "inducers". Overall, 15 of the 16 hits tested caused a quantitative decrease in production of RNAIII, as assayed by Northern Blot, whereas an inducer (Compound 6) allowed overexpression of RNAIII (See examples and Figures 14 and 15). In addition, several compounds lowered the signal from α -hemolysin (an Agr positively-controlled exotoxin) in both a plate assay and immunoblot (Figures 16 and 17), and lowered signal from staphylococcal enterotoxin B (SEB) in immunoblot analysis (Figure 17). Accordingly, some putative Agr inhibitors were thus found to increase the level of expression of protein A which is an Agr negatively-controlled surface protein (Figure 18). In the case of RNAIII, both ethidium bromide staining of the gel prior to transfer and the use of a second probe (for RecA) establish that essentially equivalent amounts of RNA were loaded in each lane; quantitation of recA signal is

used for normalization of small differences in load. Glycerol Monolaureate (GML) is a surfactant and putative signal transduction inhibitor that is known to lower the production of several toxins in an Agr independent manner (Schlievert et al., *Antimicrob. Agents Chemother.* 36:626-631 (1992); Projan et al., *J. Bacteriol.* 176:4204-4209 (1994)). In Figures 14 and 15, the effect of GML and the inducer are shown for comparison. Furthermore, GML, which showed no effect on RNAIII, did not qualify as a hit in the screen.

One screen hit (compound 18) showed synergy with methicillin against a methicillin resistant and PBP2a producing strain of *S. aureus* (Figure 19). In addition, a subset of Agr hits that affect chemotaxis or sporulation has been identified. In the case of chemotaxis, one particular hit (compound 11) which is not a chemi-attractant or -repellant itself, deregulated chemotaxis and enhanced swarm diameters of *S. typhimurium* on ribose-containing swarm agar plates. The sporulation hits (compounds 3 and 14) lowered the percent sporulation generated by *B. subtilis* strain 168 by more than 50% compared to untreated controls.

Table 4 summarizes secondary tests performed with the compounds identified as active compounds (putative Agr inhibitors), or as inducer (compound 6, data for compound 5 not shown) in the P3- β -lactamase fusion screen. As discussed above, hits were characterized by various secondary assays to demonstrate effects of compounds on the global regulator Agr (RNAIII, alpha-hemolysin, lipase, V8 protease, and protein A

expression), and on other two-component regulatory systems (sporulation, chemotaxis and specific drug synergy tests).

Finally, as an example, Figure 20 confirms that the effect of hits observed in secondary tests are also applicable to clinical strains of *S. aureus*. RNAIII expression in several clinical strains of *S. aureus* was shown to be greatly reduced in the presence of compound 10.

10

Table 4								
Properties of some confirmed agr screen hits in various tests involving the global regulator or other two component regulatory systems								
Hit	RNAIII	Alpha-Hemolysin	Lipase	V8 Protease	Protein A	Sporulation	Chemotaxis	Drug Synergy
1	+	++	++	N	N	N	N	nd
2	++	++	++	+	[++]	N	N	nd
3	+	++	+	+	[++]	++	N	N
4	N	++	+	+	nd	N	N	N
6	[++]	[++]	N	[++]	[+]	N	N	N
7	++	++	N	N	nd	N	N	N
8	++	N	N	+	nd	N	N	N
9	+	++	+	+	nd	N	N	N
10	++	++	+	+	nd	N	N	nd
11	++	++	+	+	nd	N	[++]	nd
12	++	++	+	N	nd	N	nd	N
13	++	++	++	+	nd	N	N	N
14	++	++	++	+	nd	++	N	nd
15	+	++	++	+	nd	N	N	N
16	++	++	++	N	nd	N	N	N
17	++	++	+	N	nd	N	N	N
18	++	++	++	+	nd	N	N	++

- 5 ++: Compounds that reduce expression of RNAIII, alpha-hemolysin, lipase, or V8 protease by more than 50% compared to expression levels observed in untreated wild type controls (Agr+ *S. aureus* cells). Also indicated by an identical symbol are compounds that decrease *B. subtilis* sporulation by more than 50% and compounds that act synergistically in combination with drugs such as methicillin against *S. aureus*.
- 10 +: Compounds that reduce expression of RNAIII, alpha-hemolysin, lipase, or V8 protease by 25% to 50% compared to production levels observed in untreated wild type controls (Agr+ *S. aureus* cells).
- 15 [++]: Compounds that increase expression of RNAIII, alpha-hemolysin, lipase, or V8 protease by more than 50% compared to expression levels observed in untreated wild type controls (Agr+ *S. aureus* cells). Also indicated by an identical symbol
- 20 are compounds that increase *S. aureus* protein A expression to at least 50% of the level of expression observed in Agr- mutant cells and compounds that increase *Salmonella typhimurium* chemotaxis compared to controls.
- 25 Ñ: Compounds lacking significant effect in the indicated tests.
- nd: Not determined.

H. Inducers - Identification and Use

- 30 As shown in Tables 3 and 4 above, two of the compounds (Compounds 5 and 6) identified using the P3- β -lactamase screen showed inducer rather than inhibitor activity, as defined by the screen algorithm (see brief description for Fig. 5). As shown in Table 4, Compound 6 increased the level of activity of certain other

cellular activities. Additional inducers can be identified by testing of analogs of identified inducers, as well as by additional screening of compounds. Inducer compounds, in addition to the inhibitor
5 compounds, are useful, and are also within the scope of this invention.

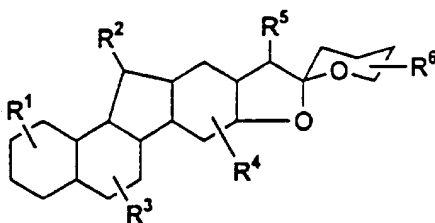
In certain regulatory systems, an inducer of a particular signal inhibits a particular pathogenesis-related response(s). In such cases, such an inducer of
10 a regulator which decreases a pathogenic response may be used in a manner similar to an inhibitor of a regulator which decreases a pathogenic response. Thus, such inducers can be utilized in therapeutic compositions and methods of treating.

15 In addition, inducer compounds can be used as evaluation controls in comparison with inhibitor compounds, and are, therefore, useful test reagents in the methods of this invention.

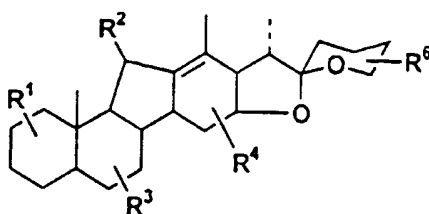
Further, as described above, in the *agr* system
20 (as well as in other regulatory systems), a peptide or protein factor acts as a natural inducer of a particular step or steps in a pathogenic response(s) (see Guangyong et al, 1995, *Proc. Natl. Acad. Sci. USA* 92:12055-12059; Balaban & Novick, 1995, *Proc. Natl. Acad. Sci. USA*
25 92:1619-1623). Balaban & Novick indicated that sera containing antibodies against the octapeptide inducer block activation of the *agr* response. Therefore, antibodies against small molecule inducers which mimic the binding of a natural inducer can also bind to the
30 natural inducer and block a pathogenic response such as

the Agr response. The generation of such antibodies by a small molecule inducer may, in fact, be more efficient than the generation of antibodies by a peptide or protein inducer since it would be expected that such peptides would be rapidly degraded. Therefore, inactive analogs of inducers that would still bind to, or interact with microbial response regulators, and that would compete with the microbe's natural inducers, can be used to generate an immune response in the host and/or to competitively abolish stimulating effects by the natural inducers. Either of these processes would interfere with the development of and/or effects of a particular microbial infection.

As indicated above, two inducer compounds (Compounds 5 and 6) were identified. Therefore, in particular embodiments, this invention includes those compounds and related active compounds described by the general structure, Structure 3, as shown below:



and the corresponding narrower general structure, Structure 3A, below:



For the compounds described by these structures, R¹-R⁶ may be the same or different, and are selected from the group consisting of H, optionally-substituted C₁-C₄ alkyl, C₁-C₄ alkenyl, C₁-C₄ alkoxy, hydroxy, and amino. The rings shown in the general structures may be saturated or unsaturated. As was described for the identified inhibitor compounds, compounds described by these general structures, related to the identified inducers, can be obtained by a variety of methods, including obtaining analogs from commercial sources, by synthesizing particular analogs, by chemical modification of the known inducers or of available analogs of such inducers, or by additional screening of compounds in appropriate libraries.

I. In vivo evaluation of agr null mutant. Using a mucin-enhanced murine peritonitis model with Balb/c mice, reduced virulence of an agr null mutant was also demonstrated. Experiments were performed as follows:

Male Balb/c mice weighing 22-25g were obtained from Charles Rivers Labs, Hollister, CA. Mice were housed 10 per cage and given free access to food and water.

Staphylococcus aureus strains ISP479C (Agr+) and ISP 546 (Agr-) (Smeltzer et al., 1993, *Inf. & Imm.* 61:919-925) were grown overnight in Tryptic Soy (TSB) broth. The following morning, they were subcultured to fresh TSB and incubated for 4-5h. Cells were washed twice with PBS and adjusted to the desired concentration by correlation of absorbency at O.D. 600 nm with predetermined plate counts.

Mice were challenged by the intraperitoneal route with 0.5 mL of bacterial suspension in 7% hog-gastric mucin. Animals were observed for 72 h. Table 5 outlines results of such in vivo studies. These data show the difference in virulence of Agr+ and Agr- *S. aureus*, and hence what may be possible to achieve with a compound that inhibits the Agr response.

Table 5		
<i>In Vivo</i> Effects of Agr+ & Agr- <i>Staphylococcus aureus</i> Strains in Mice		
	Strain ISP479C (Agr+)	Strain ISP547 (Agr-)*
LD50 (cfu):	2.3×10^6	4.3×10^6
Challenge dose of 10^6 cfu:	All mice died	All mice lived

5 *Note: A challenge dose of 10^6 cfu was treatable with
vancomycin (3.15 mg/kg).

J. Pharmaceutical Applications

The compositions containing inhibitors of
global regulators of pathogenesis genes can be
10 administered for prophylactic and/or therapeutic
treatments. In therapeutic applications, the
compositions are administered to a patient already
suffering from an infection from bacteria (similarly
for infections by other microbes), in an amount
15 sufficient to cure or at least partially arrest the
symptoms of the infection. An amount adequate to
accomplish this is defined as "therapeutically
effective amount or dose." Amounts effective for this
use will depend on the severity and course of the

infection, previous therapy, the patient's health status and response to the drugs, and the judgment of the treating physician. In prophylactic applications, compositions containing the compounds of the invention are administered to a patient susceptible to, or otherwise at risk of, a particular infection. Such an amount is defined to be a "prophylactically effective amount or dose." In this use, the precise amounts again depend on the patient's state of health, weight, and the like. However, generally, a suitable effective dose will be in the range of 0.1 to 10000 milligrams (mg) per recipient per day, preferably in the range of 10-5000 mg per day. The desired dosage is preferably presented in one, two, three, four, or more subdoses administered at appropriate intervals throughout the day. These subdoses can be administered as unit dosage forms, for example, containing 5 to 1000 mg, preferably 10 to 100 mg of active ingredient per unit dosage form. Preferably, the compounds of the invention will be administered in amounts of between about 2.0 mg/kg to 25 mg/kg of patient body weight, between about one to four times per day.

Once improvement of the patient's conditions has occurred, a maintenance dose is administered if necessary. Subsequently, the dosage or the frequency of administration, or both, can be reduced, as a function of the symptoms, to a level at which the improved condition is retained. When the symptoms have been alleviated to the desired level, treatment can cease. Patients can, however, require intermittent

treatment on a long-term basis upon any recurrence of the disease symptoms.

In preferred embodiments of the pharmaceutical compositions, and treatment methods, the inhibitor has a structure as shown by one of the general structures, Structures 1-2 and 4-14, including Structures 1A, 2A, 2B, 4A, 5A, 6A, 7A, 8A, 9A, 10A, 11A, 12A, 13A, and 14A. These general structures correspond to the active inhibitor screening hits as shown in the description of Figures 9-12.

In the case of compounds having Structure 1 or 1A, the core structure having two fused 6-member rings and a hydroxy substituent is termed the hydronaphthalene system; the rings of this system may contain from zero to five double bonds. R^1 , R^2 , and R^3 may be the same or different, and are selected from the group consisting of H, optionally-substituted C_1 - C_4 alkyl, C_1 - C_4 alkenyl, C_1 - C_4 alkoxy, hydroxy, or amino. R^4 is an optionally-branched, saturated or unsaturated hydrocarbon chain containing up to ten carbon atoms. If unsaturated, the chain contains from one to four double bonds.

For compounds having Structure 2, 2A, or 2B, R^1 , R^2 , and R^3 may be the same or different, and are selected from the group consisting of H, optionally-substituted C_1 - C_4 alkyl, C_1 - C_4 alkenyl, C_1 - C_4 alkoxy, hydroxy, or amino. R^4 is selected from the group consisting of H, C_1 - C_4 alkyl, C_1 - C_4 alkenyl, C_1 - C_4 alkoxy, or halogen. The rings may contain single or double bonds.

For compounds having Structure 4 or 4A, R¹ is selected from the group consisting of H, C₁-C₄ alkoxy, aryloxy, araalkoxy, C₁-C₄ alkyl, and aryl. R² and R³ may be the same or different, and are selected from the group consisting of H, optionally-substituted, optionally-branched C₁-C₄ alkyl, C₁-C₄ alkenyl, aryl, or araalkyl. In specific embodiments, R² and R³, in combination with the groups to which they are attached form known natural and unnatural amino acids. In other embodiments, R² and R³ do not form natural or unnatural amino acids.

For compounds having Structures 5 or 5A, R¹, R², and R³ may be the same or different, and are selected from the group consisting of H, C₁-C₄ alkyl, C₁-C₄ alkenyl, C₁-C₄ alkoxy, amino, hydroxy, trifluoromethyl, and halogen. R⁴ is selected from the group consisting of H and C₁-C₄ alkyl.

For compounds having Structure 6 or 6A, R¹ and R² may be the same or different, and are selected from the group consisting of H, C₁-C₄ alkyl, C₁-C₄ alkenyl, C₁-C₄ alkoxy, amino, hydroxy, trifluoromethyl, and halogen. R³ is selected from the group consisting of H, optionally-substituted C₁-C₄ alkyl, C₁-C₄ alkenyl, C₁-C₄ alkoxy, hydroxy, and amino. R⁴ is selected from the group consisting of H, optionally-substituted C₁-C₄ alkyl, C₁-C₄ alkenyl, and C₁-C₄ alkoxy. R⁵ is selected from the group consisting of H and C₁-C₄ alkyl.

For compounds having Structure 7 or 7A, R¹ and R² may be the same or different and R¹ and R² are selected from the group consisting of H, C₁-C₄ alkyl,

C₁-C₄ alkenyl, C₁-C₄ alkoxy, amino, hydroxy, trifluoromethyl and halogen. X is selected from the group consisting of O, N and CH₂, and R¹ is selected from the group consisting of optionally-substituted C₁-C₄ alkylamino and C₁-C₄ alkylimino.

For compounds having Structure 8 or 8A, R¹ and R² may be the same or different and R¹ and R² are selected from the group consisting of H, C₁-C₄ alkyl, C₁-C₄ alkenyl, C₁-C₄ alkoxy, amino, hydroxy, trifluoromethyl and halogen. R³ and R⁴ may be the same or different and R³ and R⁴ are selected from the group consisting of H and C₁-C₄ alkyl.

For compounds having Structure 9 or 9A, R¹ and R² may be the same or different and R¹ and R² are selected from the group consisting of H, C₁-C₄ alkyl, C₁-C₄ alkenyl, C₁-C₄ alkoxy, amino, hydroxy, trifluoromethyl and halogen. R³ and R⁴ may be the same or different and R³ and R⁴ are selected from the group consisting of H and C₁-C₄ alkyl.

For compounds having Structure 10 or 10A, R¹ and R² may be the same or different and R¹ and R² are selected from the group consisting of H, C₁-C₄ alkyl, C₁-C₄ alkenyl, C₁-C₄ alkoxy, amino, hydroxy, trifluoromethyl and halogen. R³ is selected from the group consisting of H and C₁-C₄ alkyl.

For compounds having Structure 11 or 11A, R¹, R², and R³ may be the same or different and R¹, R², and R³ are selected from the group consisting of H and optionally-substituted C₁-C₁₂ alkyl, C₁-C₁₂ alkenyl, and C₁-C₁₂ alkoxy. In specific embodiments, R¹, R², and R³

together form a ring of up to 16 atoms.

For compounds having Structure 12 or 12A, R¹ and R² may be the same or different and R¹ and R² are selected from the group consisting of H, C₁-C₄ alkyl, C₁-C₄ alkenyl, C₁-C₄ alkoxy, amino, nitro, hydroxy, trifluoromethyl and halogen.

For compounds having Structure 13 or 13A, R¹ is selected from the group consisting of H, optionally-substituted C₁-C₄ alkyl, C₁-C₄ alkenyl, C₁-C₄ alkoxy, hydroxy, and amino. R² is selected from the group consisting of H and optionally-substituted C₁-C₄ alkyl, C₁-C₄ alkenyl, and C₁-C₄ alkenoyl. R³ is selected from the group consisting of H, C₁-C₄ alkyl, C₁-C₄ alkenyl, C₁-C₄ alkoxy, amino, hydroxy, trifluoromethyl and halogen.

For compounds having Structure 14 or 14A, R¹, R², and R³ may be the same or different and R¹, R², and R³ are selected from the group consisting of H, C₁-C₄ alkyl, C₁-C₄ alkenyl, C₁-C₄ alkoxy, amino, hydroxy, trifluoromethyl and halogen.

As used in the descriptions herein, the term "alkyl" refers to an aliphatic hydrocarbon, preferably a saturated hydrocarbon, either unbranched or branched. Preferably the alkyl group contains one to 12 carbons, more preferably from one to four carbons, such as, e.g., methyl, ethyl, n-propyl, iso-propyl, n-butyl, sec-butyl, iso-butyl and tert-butyl. The alkyl group may be optionally substituted with one or more functional groups which are attached commonly to such chains, preferably hydroxyl, bromo, fluoro, chloro,

iodo, mercapto or thio, cyano, alkylthio, heterocycle, aryl, heteroaryl, carboxyl, carboalkoyl, alkyl, alkenyl, nitro, amino, alkoxyl, amido, and the like.

The term "alkenyl" denotes an alkyl group as
5 defined above having at least one double bond, such as, e.g., vinyl, allyl, 1-propenyl, isopropenyl, 1-butenyl, 2-butenyl, 3-butenyl, methallyl, or 1,1-dimethylallyl.

The term "alkoxyl" denotes the group -OR, where R is alkyl or alkenyl as defined above, such as
10 methoxy, ethoxy, n-propoxy, iso-propoxy, n-butoxy, sec-butoxy, iso-butoxy, or tert-butoxy and the like.

The term "hydroxy" refers to the group -OH.

The term "amino" refers to a group -N(R)R', in which R and R' are preferably H, but may optionally be
15 independently substituted, for example with alkyl, aryl, or acyl groups.

The term "halogen" refers to a group which is selected from fluoro, chloro, bromo, and iodo, preferably fluoro or chloro.

20 The term "aryl" refers to a chain of atoms which form at least one aromatic ring, with the group indicated as "-Ar". The aromatic ring is preferably formed of carbon atoms, but may include one or more hetero atoms. The aryl group, preferably on the
25 aromatic ring, may optionally be substituted with groups commonly attached to such chains, for example, hydroxy, halo, alkyl, alkenyl, thio, nitro, amino and the like.

The term "aryloxy" denotes a group -OAr, where
30 Ar is aryl as defined above.

The term "araalkoxy" refers to a group -ORAr, in which R is an alkyl or alkenyl group and Ar is aryl as defined above.

The term "araalkyl" refers to a group -RAR, in which R is alkyl and Ar is aryl.

The term "alkylamino" refers to a group $-N(R^1)R^2$, in which at least one of the R groups is an alkyl group, and one or both R groups may be optionally substituted.

Similarly, the term "alkylimine" indicates a group $-N=R$, in which the R group is a branched or unbranched alkyl group which is optionally substituted, preferably with halo or sulfhydryl.

The term "nitro" refers to a group $-NO_2$.

The term "alkenoyl" denotes a group $-C(O)R$, in which R is an optionally substituted, branched or unbranched alkenyl group.

K. Administration

Although it is possible to administer the inhibitor alone, it is preferable to present an inhibitor as part of a pharmaceutical composition containing the active inhibitor compound and a carrier or excipient. In addition, as noted above, in some cases, the effect of administering an inhibitor of a global regulator of pathogenesis genes will be to make infecting bacteria more accessible to another antibacterial agent, or otherwise to increase the effectiveness of the administration of such other antibacterial agent. Therefore, in some cases it will

be advantageous to administer an inhibitor of a global regulator of pathogenes or of other regulatory pathways in combination (either simultaneously or serially) with another traditional antibacterial agent, preferably one
5 which has bacteriocidal effects.

The formulations of the present invention preferably contain at least one inhibitor of a global regulator of pathogenesis genes or other regulatory pathways and one or more pharmaceutically or
10 therapeutically acceptable carriers or excipients. The inhibitor compound is in such amount that the combination constitutes a pharmaceutically or therapeutically effective dose or amount. The compounds can be prepared as pharmaceutically
15 acceptable salts (i.e., non-toxic salts which do not prevent the compound from exerting its effect).

Carriers or excipient can be used to facilitate administration of the compound, for example, to increase the solubility of the compound. Solid
20 carriers include, e.g., starch, lactose, dicalcium phosphate, microcrystalline cellulose, sucrose, and kaolin, and optionally other therapeutic ingredients. Liquid carriers include, e.g., sterile water, saline, buffers, polyethylene glycols, non-ionic surfactants,
25 and edible oils such as corn, peanut and sesame oils, and other compounds described e.g., in the MERCK INDEX, Merck & Co., Rahway, NJ. In addition, various adjuvants such as are commonly used in the art may be included. For example: flavoring agents, coloring
30 agents, preservatives, and antioxidants, e.g., vitamin

E, ascorbic acid, BHT and BHA. Various other considerations are described, e.g., in Gilman et al. (eds) (1990) *Goodman and Gilman's: The Pharmacological Basis of Therapeutics*, 8th Ed., Pergamon Press.

- 5 Methods for administration are discussed therein, e.g., for oral, intravenous, intraperitoneal, or intramuscular administration, subcutaneous, topically, and others.

- These pharmaceutical compositions can be in a variety of forms. These include, for example, solid, semi-solid and liquid dosage forms, such as tablets, pills, powders, liquid solutions or suspensions, liposomes, injectable and infusible solutions. The preferred form depends on the intended mode of administration and therapeutic application. For some compounds a pharmacologically acceptable salt of the compound will be used to simplify preparation of the composition. Preferred salts include sodium, potassium, arginine, glycine, alanine, threonine.
- 15
20 These are prepared, preferably, in water suitably mixed with a surfactant such as hydroxypropylcellulose.

- The embodiments herein described are not meant to be limiting to the invention. Those of skill in the art will appreciate the invention may be practiced by using numerous compounds and by numerous methods all within the breadth of the claims. In particular, while the above description of the invention concentrates on bacterial pathogenesis, the invention also applies to global regulators of pathogenesis genes in other
- 25

pathogenic microbes.

Other embodiments are within the following claims.

Claims

What we claim is:

1. A method of treating a bacterial infection in a mammal, comprising administering to said
5 mammal a therapeutically effective amount of an inhibitor of a global regulator of pathogenesis genes.
2. The method of claim 1, wherein said inhibitor is active against a *Staphylococcal* global regulator of pathogenesis genes.
- 10 3. The method of claim 2, wherein said *Staphylococcal* global regulator is from *Staphylococcus aureus*.
4. The method of claim 2, wherein said global regulator is encoded by the *agr* locus or a
15 homolog thereof.
5. The method of claim 3, wherein said global regulator is RNAIII.
6. The method of claim 3, wherein said inhibitor reduces the autoregulation of the *agr* locus.
- 20 7. The method of claim 5, wherein said inhibitor reduces the expression of RNAIII.
8. The method of claim 2, wherein said

global regulator is encoded by the *xpr* gene or a homolog thereof.

9. The method of claim 2, wherein said global regulator is encoded by the *sar* gene or a
5 homolog thereof.

10. The method of claim 2, wherein said global regulator is encoded by the *sae* gene or a homolog thereof.

11. A method of treating a bacterial
10 infection in a mammal, comprising administering to said mammal a therapeutically effective amount of an inhibitor of one or more pathogenesis factors modulated by *agr*, *sar*, *sae*, or *xpr*.

12. A method of prophylactic treatment of a
15 mammal, comprising administering to a mammal at risk of an infection a therapeutically effective amount of an inhibitor of a global regulator of pathogenesis genes.

13. The method of claim 12, wherein said inhibitor is active against a *Staphylococcal* global
20 regulator of pathogenesis genes.

14. The method of claim 13, wherein said *Staphylococcal* global regulator is from *Staphylococcus aureus*.

15. The method of claim 13, wherein said global regulator is encoded by the agr locus or a homolog thereof.

16. The method of claim 14, wherein said
5 global regulator is RNAIII.

17. The method of claim 14, wherein said inhibitor reduces the autoregulation of the agr locus.

18. The method of claim 14, wherein said inhibitor reduces the expression of RNAIII.

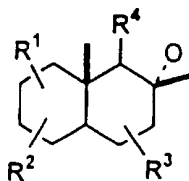
10 19. The method of claim 13, wherein said global regulator is encoded by the xpr gene or a homolog thereof.

20. The method of claim 13, wherein said global regulator is encoded by the sar gene or a
15 homolog thereof.

21. The method of claim 13, wherein said global regulator is encoded by the sae gene or a homolog thereof.

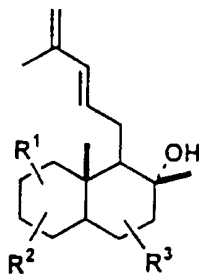
22. A method of prophylactic treatment of a
20 mammal, comprising administering to a mammal at risk of a bacterial infection a therapeutically effective amount of an inhibitor of one or more pathogenesis factors modulated by agr, sar, sae, or xpr.

23. The method of any of claims 1, 2, 11, 12, or 22, wherein said inhibitor has Structure 1, namely:

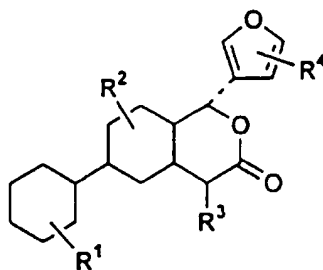


wherein R^1 , R^2 , and R^3 may be the same or different
 5 and R^1 , R^2 , and R^3 are selected from the group
 consisting of H, optionally-substituted C_1 - C_4 alkyl, C_1 - C_4 alkenyl, C_1 - C_4 alkoxy, hydroxy, and amino; and
 R^4 is a branched or unbranched, saturated or
 unsaturated hydrocarbon chain containing from one to
 10 ten carbon atoms.

24. The method of claim 23, wherein said inhibitor has Structure 1A, namely:



25. The method of any of claims 1, 2, 11, 12, or 22, wherein said inhibitor has Structure 2, namely:

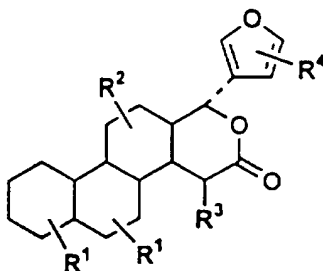


wherein R^1 , R^2 , R^3 may be the same or different and
 R^1 , R^2 , R^3 are selected from the group consisting of H,
 optionally-substituted C_1 - C_4 alkyl, C_1 - C_4 alkenyl, C_1 - C_4
 5 alkoxy, hydroxy, and amino;

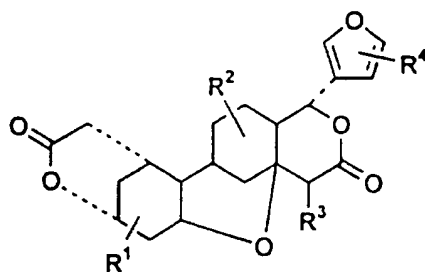
R^4 is selected from the group consisting of H, C_1 - C_4
 alkyl, C_1 - C_4 alkenyl, C_1 - C_4 alkoxy, and halogen.

26. The method of claim 25, wherein said
 inhibitor has Structure 2A, namely:

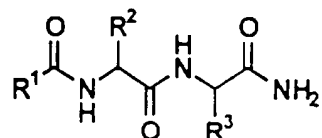
10



27. The method of claim 25, wherein said
 inhibitor has Structure 2B, namely:



28. The method of any of claims 1, 2, 11, 12, or 22, wherein said inhibitor has Structure 4, namely:

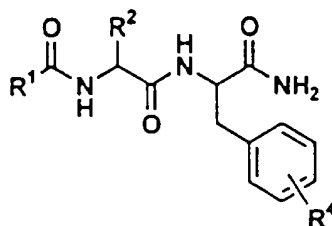


5 wherein R¹ is selected from the group consisting of H, C₁-C₄ alkoxy, aryloxy, araalkoxy, C₁-C₄ alkyl, and aryl; and

 R² and R³ may be the same or different and R² and R³ are selected from the group consisting of H,
 10 optionally-substituted, optionally-branched C₁-C₄ alkyl, C₁-C₄ alkenyl, aryl, and araalkyl.

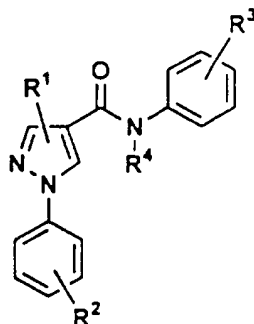
29. The method of claim 28, wherein said inhibitor has structure 4A, namely:

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wherein R⁴ is selected from the group consisting of H, C₁-C₄ alkyl, C₁-C₄ alkenyl, C₁-C₄ alkoxy, amino, hydroxy, trifluoromethyl, and halogen.

- 5 30. The method of any of claims 1, 2, 11, 12, or 22, wherein said inhibitor has Structure 5, namely:

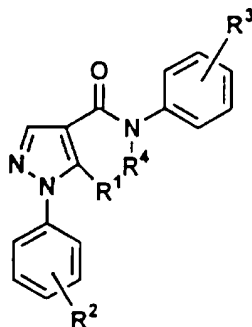


wherein R¹, R², and R³ may be the same or different, and are selected from the group consisting of H, C₁-C₄ alkyl, C₁-C₄ alkenyl, C₁-C₄ alkoxy, amino, hydroxy, trifluoromethyl, and halogen; and

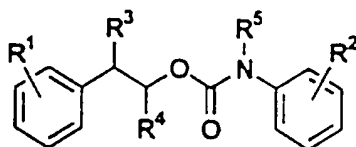
R⁴ is selected from the group consisting of H and

C₁-C₄ alkyl.

31. The method of claim 30 wherein said inhibitor has structure 5A. namely:



5 32. The method of any of claims 1, 2, 11, 12, or 22, wherein said inhibitor has Structure 6, namely:



wherein R¹ and R² may be the same or different, and are selected from the group consisting of H, C₁-C₄ alkyl, C₁-C₄ alkenyl, C₁-C₄ alkoxy, amino, hydroxy, trifluoromethyl, and halogen;

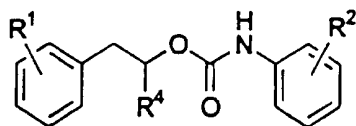
R³ is selected from the group consisting of H, optionally-substituted C₁-C₄ alkyl, C₁-C₄ alkenyl, C₁-C₄

alkoxy, hydroxy, and amino;

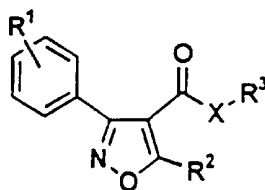
R⁴ is selected from the group consisting of H, optionally-substituted C₁-C₄ alkyl, C₁-C₄ alkenyl, and C₁-C₄ alkoxy; and

5 R⁵ is selected from the group consisting of H and C₁-C₄ alkyl.

33. The method of claim 32, wherein said inhibitor has structure 6A, namely:



10 34. The method of any of claims 1, 2, 11, 12, or 22, wherein said inhibitor has Structure 7, namely:



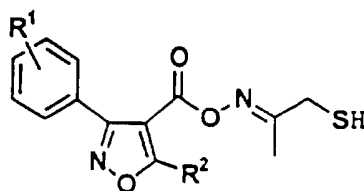
wherein R¹ and R² may be the same or different and R¹ and R² are selected from the group consisting of H, C₁-C₄ alkyl, C₁-C₄ alkenyl, C₁-C₄ alkoxy, amino, hydroxy, 15 trifluoromethyl, and halogen;

X is selected from the group consisting of O, N,

and CH₂; and

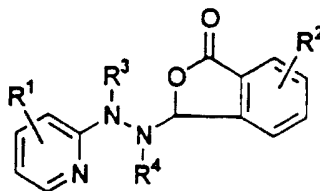
R³ is selected from the group consisting of optionally-substituted C₁-C₄ alkylamino, and C₁-C₄ alkylimino.

- 5 35. The method of claim 34 wherein said inhibitor has structure 7A, namely:



36. The method of any of claims 1, 2, 11, 12, or 22, wherein said inhibitor has Structure 8, namely:

10

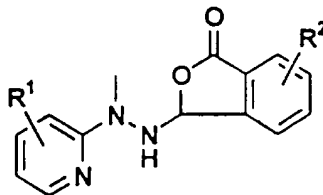


wherein R¹ and R² may be the same or different and R¹ and R² are selected from the group consisting of H, C₁-C₄ alkyl, C₁-C₄ alkenyl, C₁-C₄ alkoxy, amino, hydroxy, trifluoromethyl, and halogen; and

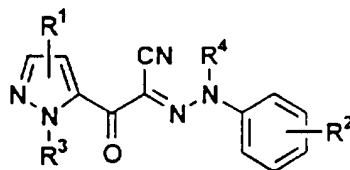
- 15 R³ and R⁴ may be the same or different and R³ and R⁴ are selected from the group consisting of H

and C₁-C₄ alkyl.

37. The method of claim 36, wherein said inhibitor has structure 8A, namely:



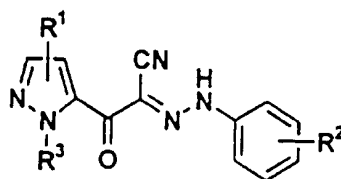
38. The method of any of claims 1, 2, 11, 12, or 22, wherein said inhibitor has Structure 9, namely:



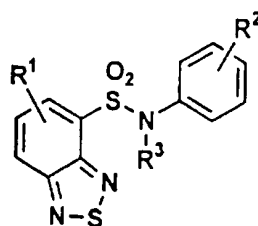
wherein R¹ and R² may be the same or different and R¹ and R² are selected from the group consisting of H, C₁-C₄ alkyl, C₁-C₄ alkenyl, C₁-C₄ alkoxy, amino, hydroxy, trifluoromethyl, and halogen; and

R³ and R⁴ may be the same or different and R³ and R⁴ are selected from the group consisting of H and C₁-C₄ alkyl.

39. The method of claim 38 wherein said inhibitor has structure 9A, namely:

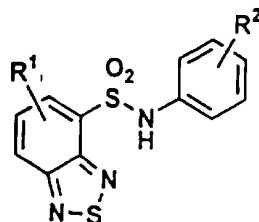


40. The method of any of claims 1, 2, 11, 12, or 22, wherein said inhibitor has Structure 10, namely:

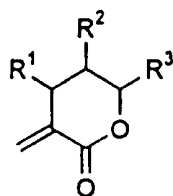


5 wherein R¹ and R² may be the same or different and
 R¹ and R² are selected from the group consisting of H,
 C₁-C₄ alkyl, C₁-C₄ alkenyl, C₁-C₄ alkoxy, amino, hydroxy,
 trifluoromethyl, and halogen, and
 R³ is selected from the group consisting of H and
 10 C₁-C₄ alkyl.

41. In the method of claim 40 wherein said inhibitor has structure 10A, namely:



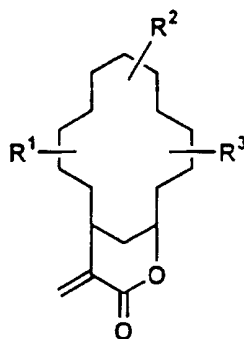
42. The method of any of claims 1, 2, 11, 12, or 22, wherein said inhibitor has Structure 11, namely:



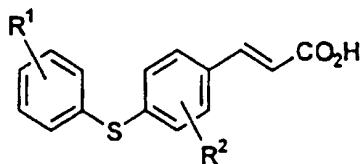
wherein R¹, R², and R³ may be the same or different
5 and R¹, R², and R³ are selected from the group
consisting of H and optionally-substituted C₁-C₁₂ alkyl,
C₁-C₁₂ alkenyl, and C₁-C₁₂ alkoxy.

43. The method of claim 42 wherein said
inhibitor has structure 11A, namely:

10

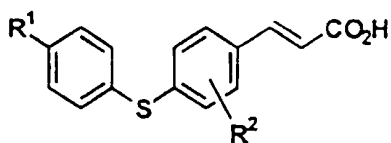


44. The method of any of claims 1, 2, 11, 12, or 22, wherein said inhibitor has Structure 12, namely:

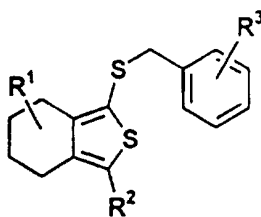


wherein R¹ and R² may be the same or different and
 R¹ and R² are selected from the group consisting of H,
 C₁-C₄ alkyl, C₁-C₄ alkenyl, C₁-C₄ alkoxy, amino, nitro,
 5 hydroxy, trifluoromethyl, and halogen.

45. The method of claim 44, wherein said
 inhibitor has structure 12A, namely:



46. The method of any of claims 1, 2, 11, 12,
 10 or 22, wherein said inhibitor has Structure 13, namely:

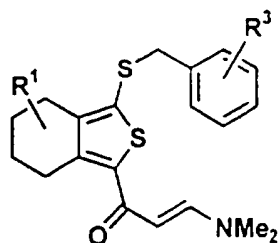


wherein R¹ is selected from the group consisting of
 H, optionally-substituted C₁-C₄ alkyl, C₁-C₄ alkenyl,
 C₁-C₄ alkoxy, hydroxy, and amino;

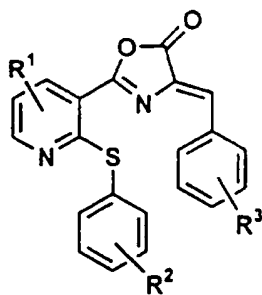
R^2 is selected from the group consisting of H, optionally-substituted C_1 - C_4 alkyl, C_1 - C_4 alkenyl, and C_1 - C_4 alkenoyl; and

R^3 is selected from the group consisting of H, C_1 - C_4 alkyl, C_1 - C_4 alkenyl, C_1 - C_4 alkoxy, amino, hydroxy, trifluoromethyl, and halogen.

47. The method of claim 46 wherein said inhibitor has structure 13A, namely:

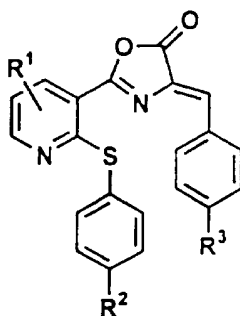


48. The method of any of claims 1, 2, 11, 12, or 22, wherein said inhibitor has Structure 14, namely:



wherein R^1 , R^2 , and R^3 may be the same or different and R^1 , R^2 , and R^3 are selected from the group consisting of H, C_1 - C_4 alkyl, C_1 - C_4 alkenyl, C_1 - C_4 alkoxy, amino, hydroxy, trifluoromethyl, and halogen.

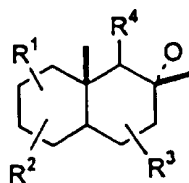
- 5 49. The method of claim 48 wherein said inhibitor has structure 14A, namely:



50. A pharmaceutical composition, comprising an inhibitor of a global regulator of pathogenesis genes and a pharmaceutically acceptable carrier.
- 10

51. A pharmaceutical composition of claim 50, wherein said inhibitor reduces the level of expression of said global regulator of pathogenesis genes.

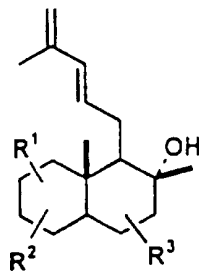
52. The pharmaceutical composition of claim 51, wherein said inhibitor has Structure 1, namely:
- 15



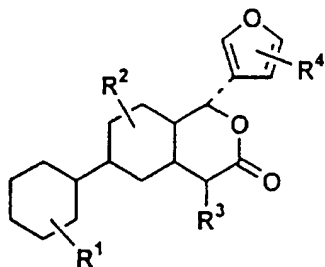
wherein R^1 , R^2 , R^3 may be the same or different and R^1 , R^2 , R^3 are selected from the group consisting of H, optionally-substituted C_1 - C_4 alkyl, C_1 - C_4 alkenyl, C_1 - C_4 alkoxy, hydroxy, and amino; and

R^4 is a branched or unbranched, saturated or unsaturated hydrocarbon chain containing from one to ten carbon atoms.

53. The pharmaceutical composition of claim 52 wherein said inhibitor has Structure 1A, namely:



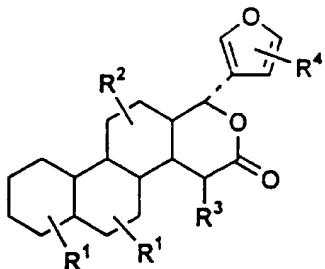
54. The pharmaceutical composition of claim 51, wherein said inhibitor has Structure 2, namely:



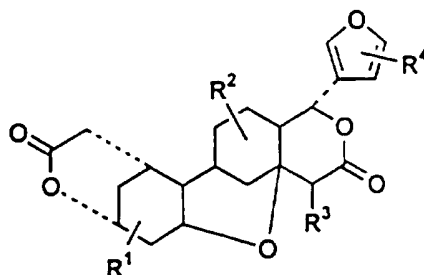
wherein R¹, R², and R³ may be the same or different and
 R¹, R², and R³ are selected from the group consisting of
 H, optionally-substituted C₁-C₄ alkyl, C₁-C₄ alkenyl, C₁-
 5 C₄ alkoxy, hydroxy, and amino; and

R⁴ is selected from the group consisting of H, C₁-C₄
 alkyl, C₁-C₄ alkenyl, C₁-C₄ alkoxy, and halogen.

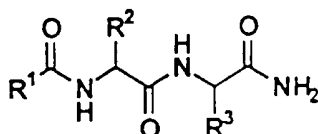
55. The pharmaceutical composition of claim
 54 wherein said inhibitor has Structure 2A, namely:



56. The pharmaceutical composition of claim
54 wherein said inhibitor has Structure 2B, namely:



57. The pharmaceutical composition of claim
51, wherein said inhibitor has Structure 4, namely:

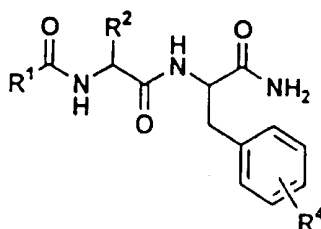


wherein R¹ is selected from the group consisting of H,
C₁-C₄ alkoxy, aryloxy, araalkoxy, C₁-C₄ alkyl, and aryl;
and

10 R¹ and R³ may be the same or different and R₂ and R₃
are selected from the group consisting of H,
optionally-substituted, optionally-branched C₁-C₄ alkyl,
C₁-C₄ alkenyl, aryl, and araalkyl.

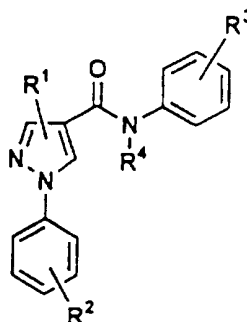
58. The pharmaceutical composition of claim
15 57 wherein said inhibitor has structure 4A, namely:

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wherein R^4 is selected from the group consisting of H, C_1-C_4 alkyl, C_1-C_4 alkenyl, C_1-C_4 alkoxy, amino, hydroxy, trifluoromethyl, or halogen.

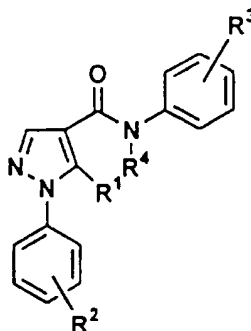
- 5 59. The pharmaceutical composition of claim 51, wherein said inhibitor has Structure 5, namely:



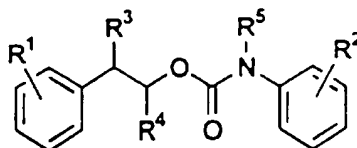
wherein R^1 , R^2 , and R^3 may be the same or different, and are selected from the group consisting of H, C_1-C_4 alkyl, C_1-C_4 alkenyl, C_1-C_4 alkoxy, amino, hydroxy, trifluoromethyl, and halogen; and

R^4 is selected from the group consisting of H and C_1-C_4 alkyl.

60. The pharmaceutical composition of claim 59, wherein said inhibitor has structure 5A, namely:



61. The pharmaceutical composition of claim 51, wherein said inhibitor has Structure 6, namely:



wherein R¹ and R² may be the same or different, and are selected from the group consisting of H, C₁-C₄ alkyl, C₁-C₄ alkenyl, C₁-C₄ alkoxy, amino, hydroxy, trifluoromethyl, and halogen;

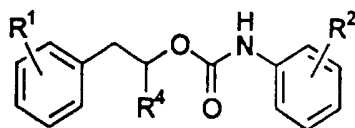
R³ is selected from the group consisting of H, optionally-substituted C₁-C₄ alkyl, C₁-C₄ alkenyl, C₁-C₄ alkoxy, hydroxy, and amino;

R⁴ is selected from the group consisting of H,

optionally-substituted C₁-C₄ alkyl, C₁-C₄ alkenyl, and C₁-C₄ alkoxy; and

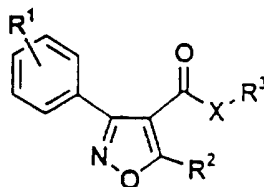
R⁵ is selected from the group consisting of H and C₁-C₄ alkyl.

- 5 62. The pharmaceutical composition of claim 61 wherein said inhibitor has structure 6A, namely:



63. The pharmaceutical composition of claim 51, wherein said inhibitor has Structure 7, namely:

10

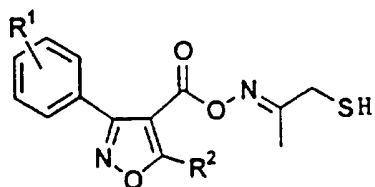


wherein R¹ and R² may be the same or different and R¹ and R² are selected from the group consisting of H, C₁-C₄ alkyl, C₁-C₄ alkenyl, C₁-C₄ alkoxy, amino, hydroxy, trifluoromethyl, and halogen;

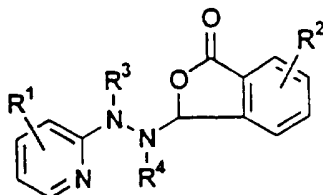
- 15 X is selected from the group consisting of O, N, and CH₂; and

R³ is selected from the group consisting of optionally-substituted C₁-C₄ alkylamino, and C₁-C₄ alkylimino.

64. The pharmaceutical composition of claim
63 wherein said inhibitor has structure 7A, namely:



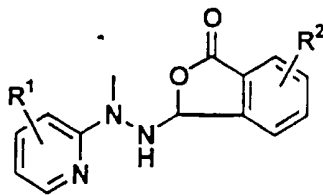
65. The pharmaceutical composition of claim
5 51, wherein said inhibitor has Structure 8, namely:



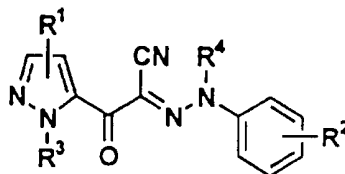
wherein R¹ and R² may be the same or different and
R¹ and R² are selected from the group consisting of H,
C₁-C₄ alkyl, C₁-C₄ alkenyl, C₁-C₄ alkoxy, amino, hydroxy,
10 trifluoromethyl, and halogen; and

R³ and R⁴ may be the same or different and
R³ and R⁴ are selected from the group consisting of H
and C₁-C₄ alkyl.

66. The pharmaceutical composition of claim
15 65 wherein said inhibitor has structure 8A, namely:



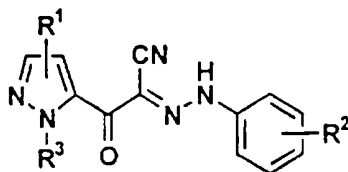
67. The pharmaceutical composition of claim 51, wherein said inhibitor has Structure 9, namely:



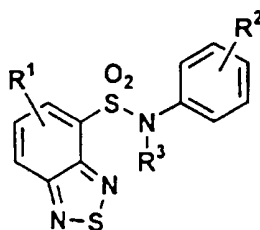
5 wherein R¹ and R² may be the same or different and R¹ and R² are selected from the group consisting of H, C₁-C₄ alkyl, C₁-C₄ alkenyl, C₁-C₄ alkoxy, amino, hydroxy, trifluoromethyl, and halogen; and

10 R³ and R⁴ may be the same or different and R³ and R⁴ are selected from the group consisting of H and C₁-C₄ alkyl.

68. The pharmaceutical composition of claim 67 wherein said inhibitor has structure 9A, namely:

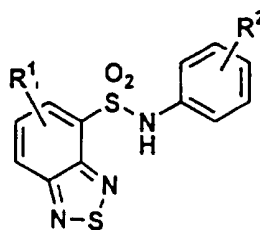


69. The pharmaceutical composition of claim 51, wherein said inhibitor has Structure 10, namely:

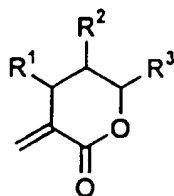


5 wherein R¹ and R² may be the same or different and
 R¹ and R² are selected from the group consisting of H,
 C₁-C₄ alkyl, C₁-C₄ alkenyl, C₁-C₄ alkoxy, amino, hydroxy,
 trifluoromethyl, and halogen; and
 R³ is selected from the group consisting of H and
 10 C₁-C₄ alkyl.

70. The pharmaceutical composition of claim 69 wherein said inhibitor has structure 10A, namely:



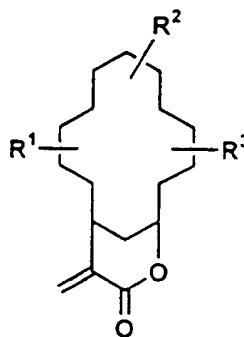
71. The pharmaceutical composition of claim 51, wherein said inhibitor has Structure 11, namely:



wherein R¹, R², and R³ may be the same or different
 5 and R¹, R², and R³ are selected from the group
 consisting of H, optionally-substituted C₁-C₁₂ alkyl, C₁-
 C₁₂ alkenyl, and C₁-C₁₂ alkoxy.

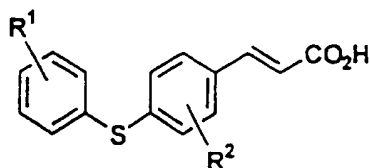
72. The pharmaceutical composition of claim 71 wherein said inhibitor has structure 11A, namely:

10



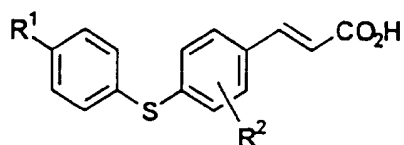
73. The pharmaceutical composition of claim 51, wherein said inhibitor has Structure 12, namely:

111

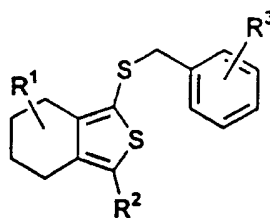


wherein R^1 and R^2 may be the same or different and R^1 and R^2 are selected from the group consisting of H, C_1 - C_4 alkyl, C_1 - C_4 alkenyl, C_1 - C_4 alkoxy, amino, nitro, hydroxy, trifluoromethyl, and halogen.

74. The pharmaceutical composition of claim 73 wherein said inhibitor has structure 12A, namely:



75. The pharmaceutical composition of claim 51, wherein said inhibitor has Structure 13, namely:

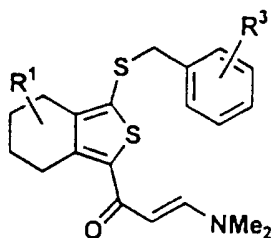


wherein R^1 is H, optionally-substituted C_1 - C_4 alkyl, C_1 - C_4 alkenyl, C_1 - C_4 alkoxy, hydroxy, or amino; and R^2 is selected from the group consisting of H and

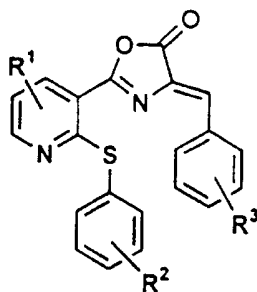
optionally-substituted C₁-C₄ alkyl, C₁-C₄ alkenyl, or C₁-C₄ alkenoyl; and

R³ is selected from the group consisting of H, C₁-C₄ alkyl, C₁-C₄ alkenyl, C₁-C₄ alkoxy, amino, hydroxy, trifluoromethyl, and halogen.

76. The pharmaceutical composition of claim 75 wherein said inhibitor has structure 13A, namely:



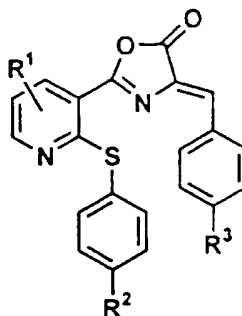
77. The pharmaceutical composition of claim 51, wherein said inhibitor has structure 14, namely:



wherein R¹, R², and R³ may be the same or different

and R¹, R², and R³ are selected from the group consisting of H, C₁-C₄ alkyl, C₁-C₄ alkenyl, C₁-C₄ alkoxy, amino, hydroxy, trifluoromethyl, and halogen.

78. The pharmaceutical composition of claim
5 77 wherein said inhibitor has structure 14A, namely:



79. The pharmaceutical composition of claim
51, wherein said global regulator of pathogenesis genes
is from a *Staphylococcal* strain.

10 80. The pharmaceutical composition of claim
79 wherein said *Staphylococcal* global regulator is from
Staphylococcus aureus.

81 The pharmaceutical composition of claim
79, wherein said global regulator is encoded by the *agr*
15 locus or a homolog thereof.

82. The pharmaceutical composition of claim

79, wherein said global regulator is encoded by the xpr gene or a homolog thereof.

83. The pharmaceutical composition of claim 79, wherein said global regulator is encoded by the sar gene or a homolog thereof.

84. The pharmaceutical composition of claim 79, wherein said global regulator is encoded by the sae gene or a homolog thereof.

85. The pharmaceutical composition of claim 80, wherein said global regulator is RNAIII.

86. The pharmaceutical composition of claim 80, wherein said inhibitor reduces the autoregulation of the agr locus.

87. A pharmaceutical composition, comprising an inhibitor of one or more agr-related regulators or pathogenesis factors and a pharmaceutically acceptable carrier.

88. A method of screening for an inhibitor of a global regulator of pathogenesis genes, comprising determining if a test compound alters the level of activity of said global regulator.

89. The method of claim 88, comprising determining if said test compound alters the level of

expression of said global regulator.

90. The method of claim 89, comprising the steps of:

a) detecting the transcriptional or translational
5 product of a hybrid DNA construct comprising a regulatory region of a gene encoding a global regulator of pathogenesis genes transcriptionally linked with a reporter gene, and

b) determining whether the amount of the
10 transcriptional or translational product of said reporter gene differs in the presence and absence of said test compound,

wherein said hybrid DNA construct is incorporated into a bacterium.

15 91. The method of claim 89, wherein said global regulator of pathogenesis genes is from a *Staphylococcus* strain.

92. The method of claim 91, wherein said
20 *Staphylococcal* global regulator is from *Staphylococcus aureus*.

93 The method of claim 91, wherein said *Staphylococcal* global regulator is encoded by the *agr* locus or a homolog thereof.

94. The method of claim 92, wherein said
25 global regulator is the RNAIII transcript.

95. The method of claim 91, wherein said global regulator is encoded by the *xpr* gene or a homolog thereof.

96. The method of claim 91, wherein said
5 global regulator is encoded by the *sar* gene or a homolog thereof.

97. The method of claim 91, wherein said global regulator is encoded by the *sae* gene or a homolog thereof.

10 98. A method for making an antibacterial agent, comprising the steps of
screening for said agent by measuring the ability of said agent to alter the level of activity of a global regulator of pathogenesis genes; and
15 synthesizing said therapeutic agent in an amount sufficient to provide said agent in a therapeutically effective amount to a patient.

99. The method of claim 98, wherein said screening further comprises
20 detecting the amount of the transcriptional or translational product of a hybrid DNA construct comprising a regulatory region of a gene encoding a global regulator of pathogenesis genes transcriptionally linked with a reporter gene, in the
25 presence and absence of said agent,
wherein said construct is incorporated into a

bacterium.

100. The method of claim 98, wherein said global regulator is from a *Staphylococcal* strain.

101. The method of claim 100, wherein said
5 global regulator is encoded by a gene selected from a gene of the *agr* locus, *xpr*, *sar*, or *sae*.

102. The method of claim 101, wherein said agent has a structure selected from the group consisting of Structures 1, 2, 4, 5, 6, 7, 8, 9, 10,
10 11, 12, 13, and 14.

103. The method of claim 98, further comprising the step of adding a pharmaceutically acceptable carrier to said agent.

FIGURE 1.

S. aureus AGR locus and response pathway
Global Regulator of Many Virulence Factors

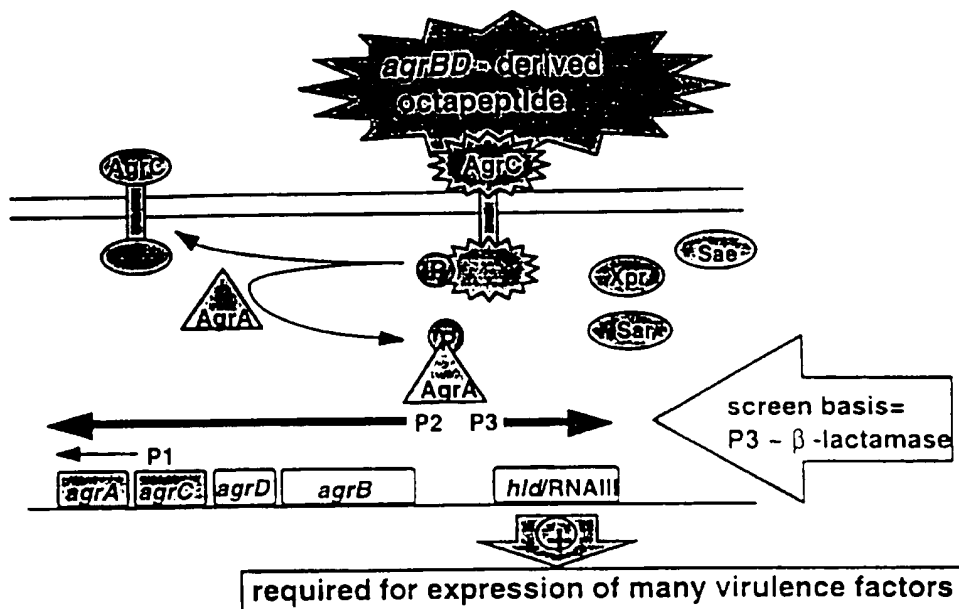
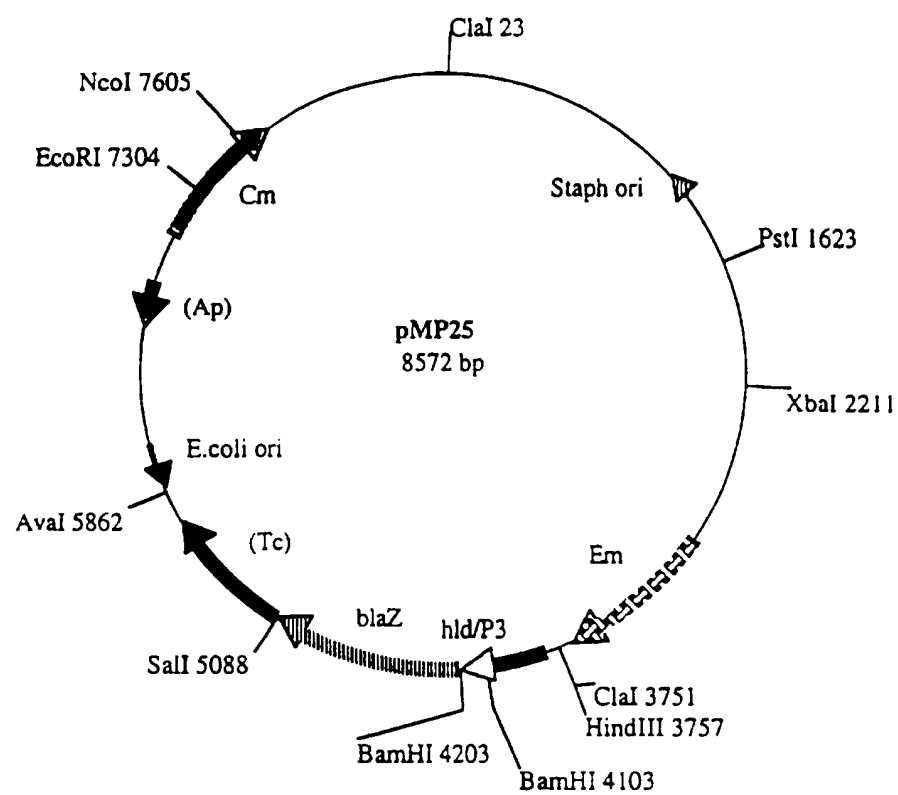


FIGURE 2.

Monitor activity of P3 with transcript fusion

 **P3** β -lactamase

FIGURE 3.

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FIGURE 4.

Temporal Regulation and Agr Dependence
of the Reporter β -Lactamase Activity Driven from the
P3 Fusion Construct in Agr+ and Agr- Strains

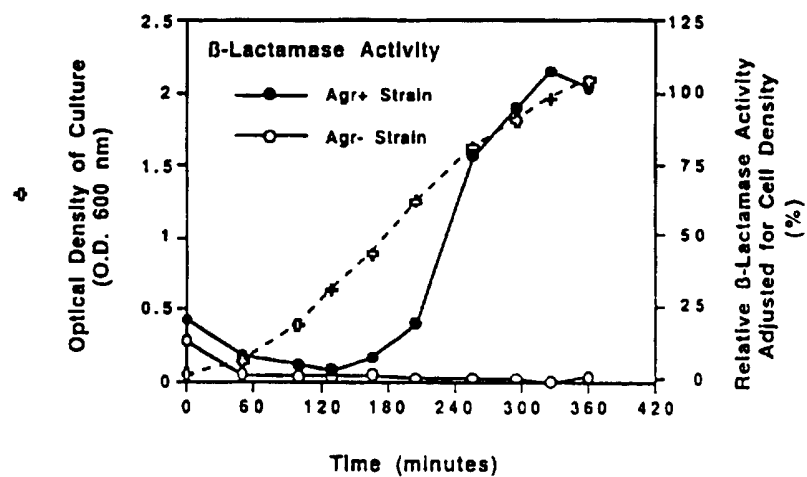


FIGURE 6.

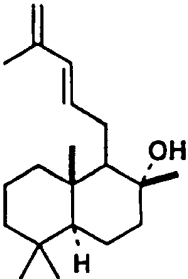
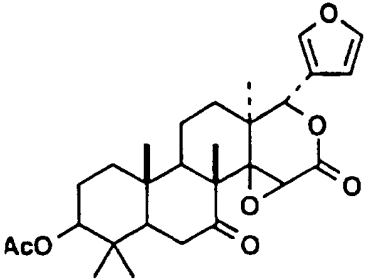
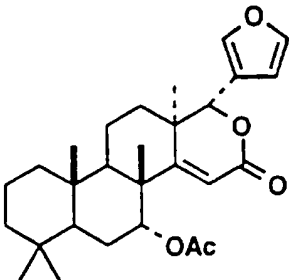
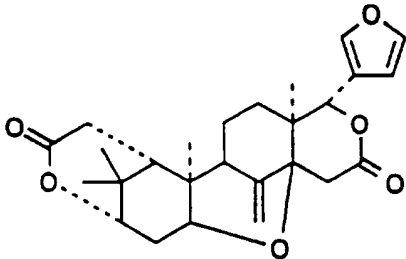
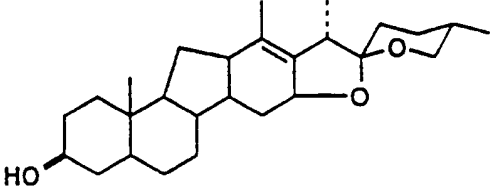
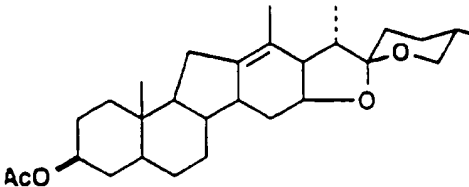
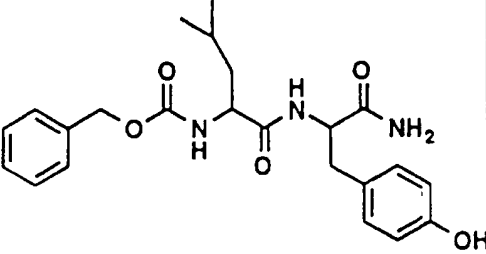
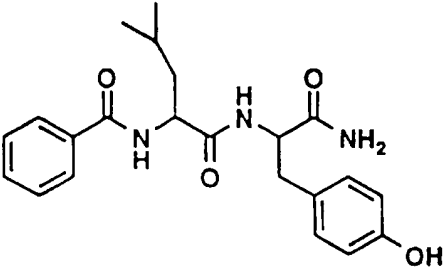
Compound 1.	Compound 2.
	
Compound 3.	Compound 4.
	
Compound 5.	Compound 6.
	
Compound 7.	Compound 8.
	

FIGURE 7

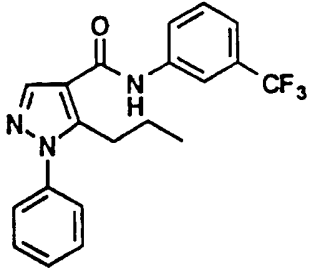
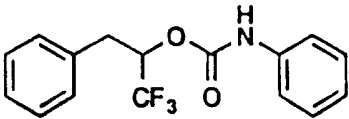
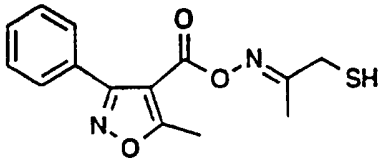
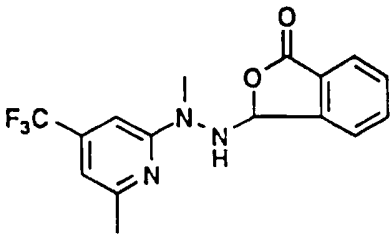
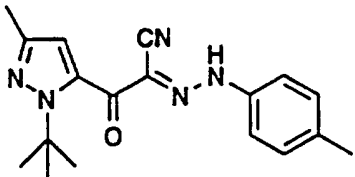
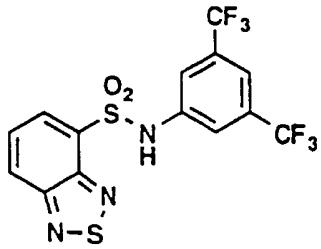
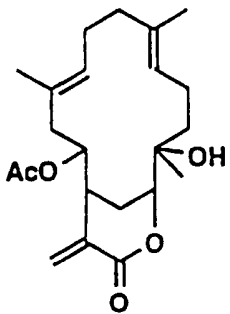
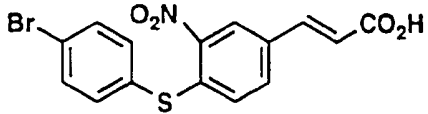
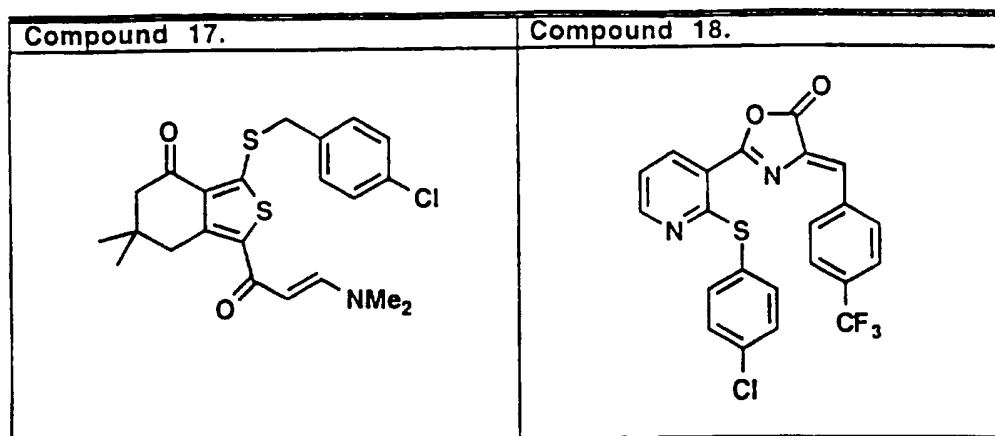
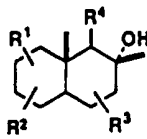
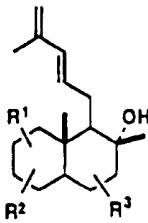
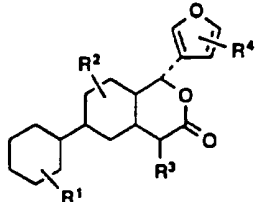
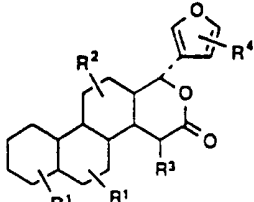
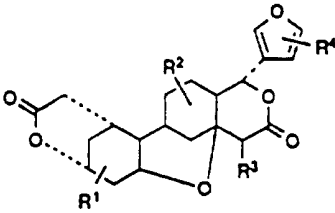
Compound 9.	Compound 10.
	
Compound 11.	Compound 12.
	
Compound 13.	Compound 14.
	
Compound 15.	Compound 16.
	

FIGURE 8.



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FIGURE 9

<u>Generic and Sub-generic Structures</u> <u>Corresponding to Screening Hits 1-18</u>	
<u>Generic Structures</u>	<u>Sub-generic Structures</u>
 <p>Structure 1 (Corresponds to Compound 1)</p>	 <p>Structure 1A</p>
 <p>Structure 2 (Corresponds to Compounds 2-4)</p>	 <p>Structure 2A (Corresponds to Compounds 2-3)</p>
	 <p>Structure 2B (Corresponds to Compound 4)</p>

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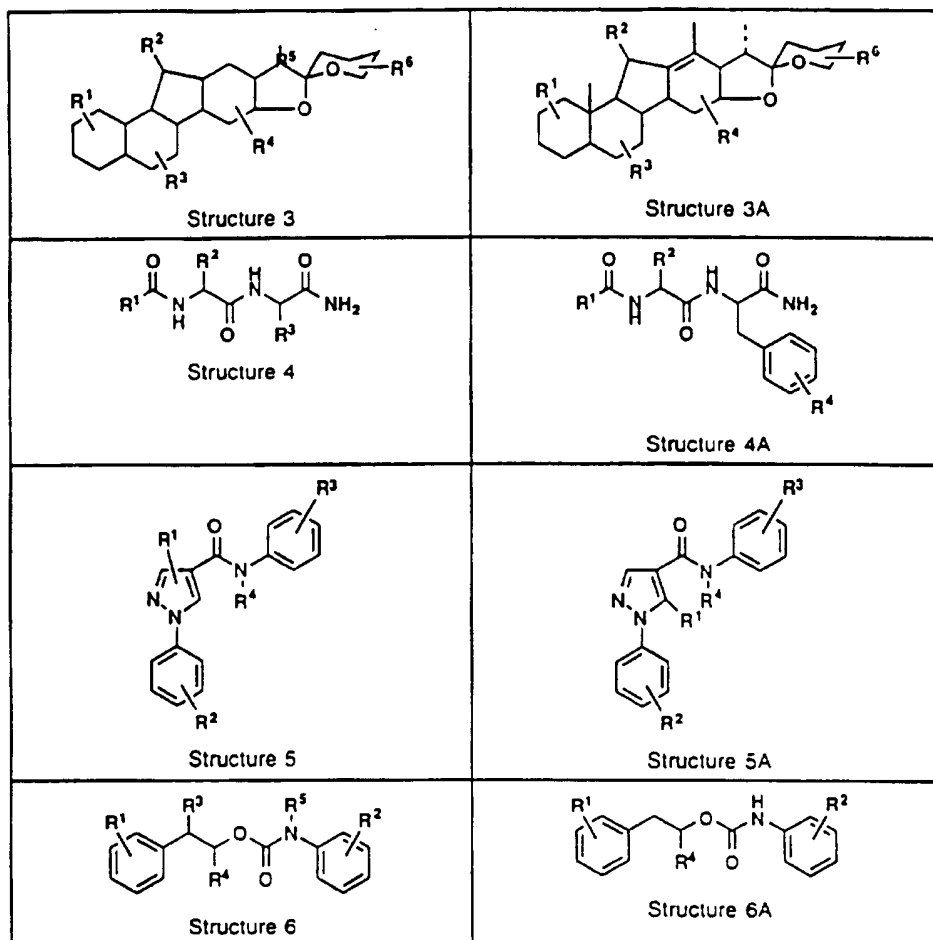
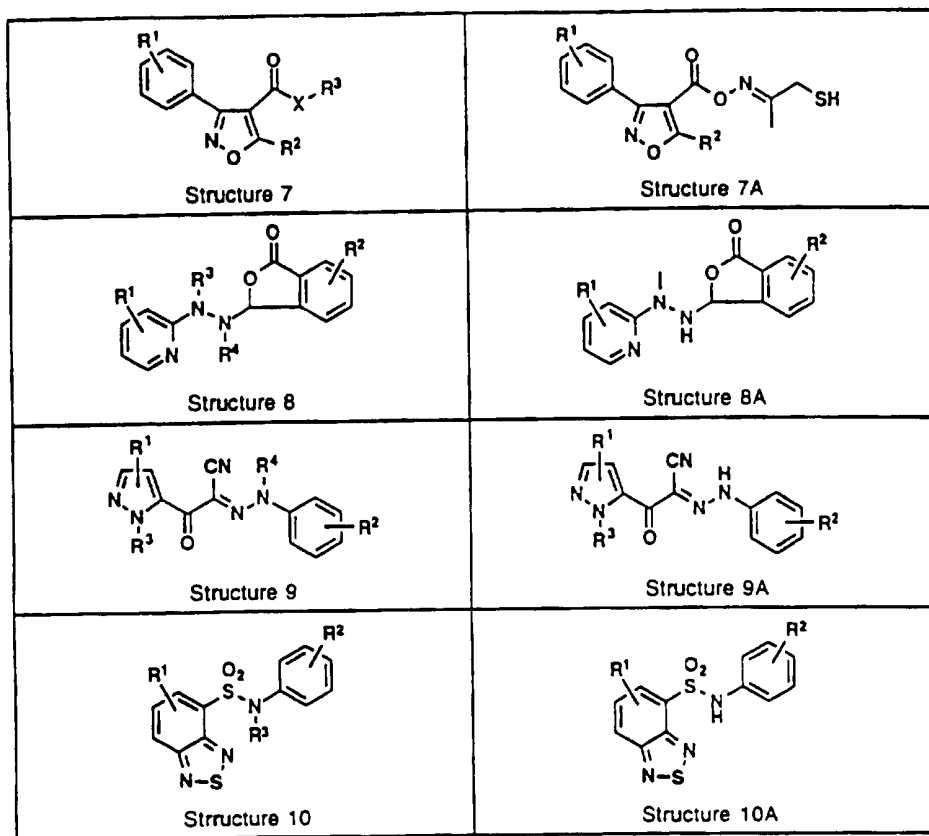
FIGURE 10

FIGURE 11

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FIGURE 12

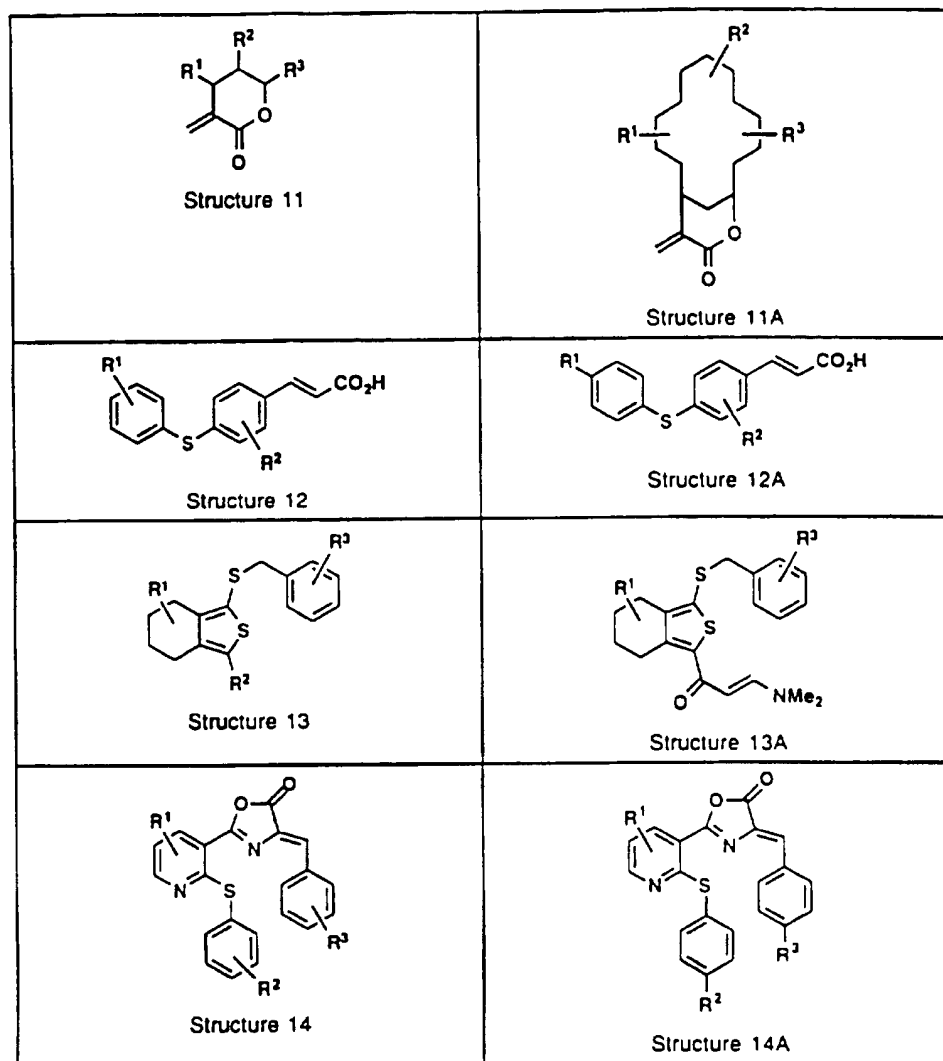


FIGURE 13.

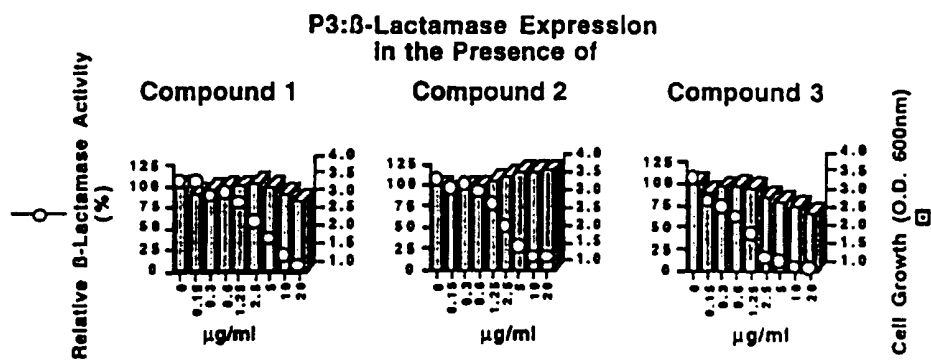


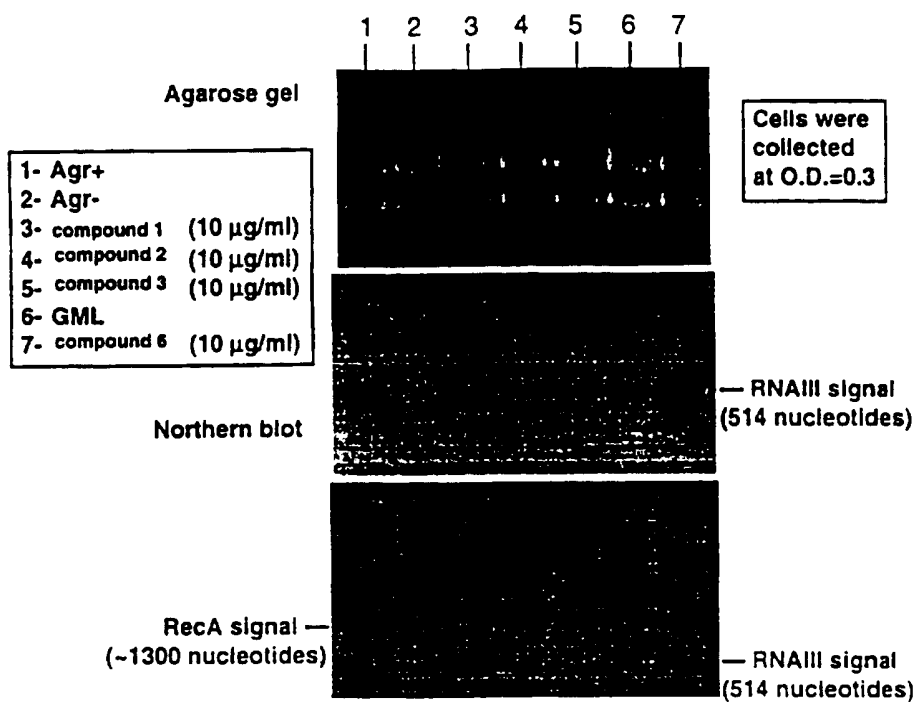
FIGURE 14.

FIGURE 15.

**RNAIII & RecA Probe Signals on
Northern Blots from *S. aureus* cells
(O.D.600nm=0.3)**

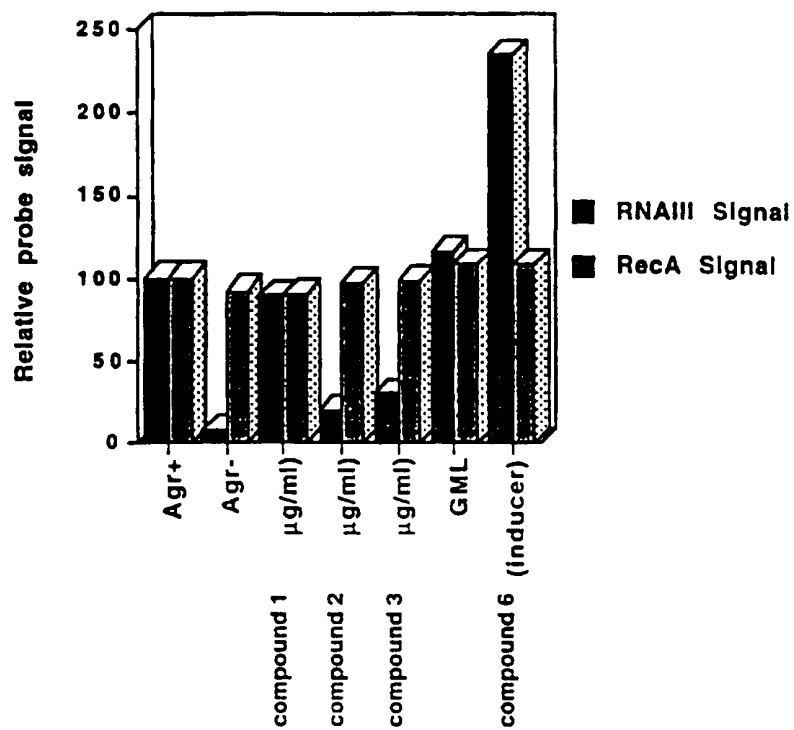


FIGURE 16.

- 1: Compound 1 (10 μ g/ml)
2: Compound 2 (20 μ g/ml)
3: Compound 2 (10 μ g/ml)
4: Compound 3 (5 μ g/ml)
5: Compound 3 (2.5 μ g/ml)
9: Clindamycin (0.5 μ g/ml)
10: Ciprofloxacin (0.06 μ g/ml)
11: Vancomycin (0.5 μ g/ml)

Agr+: Agr+ supernatants

Agr-: Agr- supernatants

NC: Media (no cells)

FIGURE 17.

**Seb and α -Hemolysin Detected in
Exoproducts Collected from *S. aureus*
Cells (O.D.=0.5-0.6)**

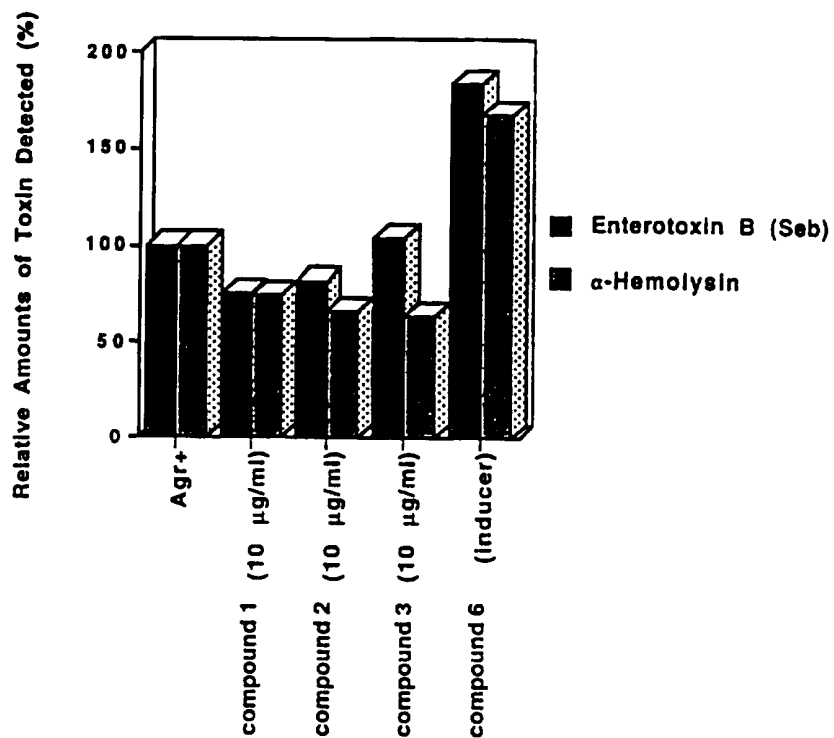
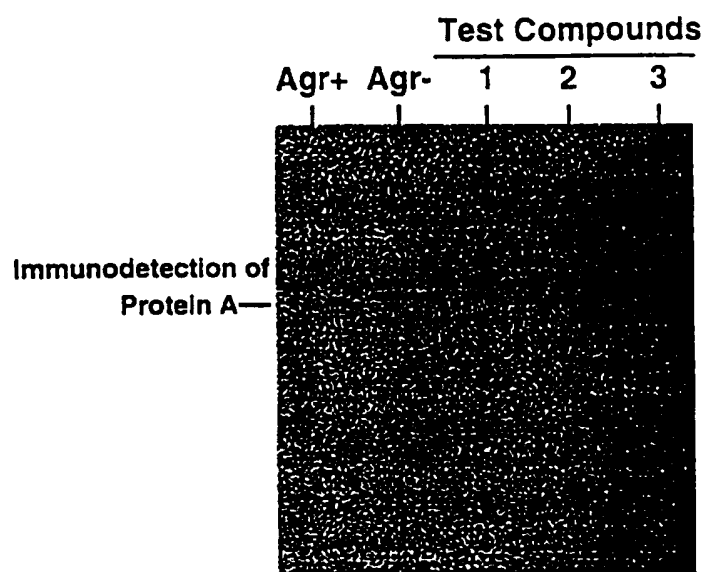


FIGURE 18.

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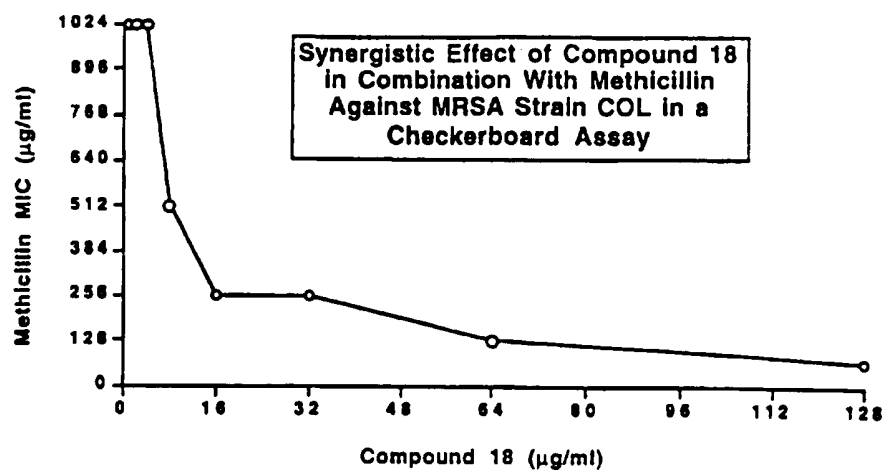
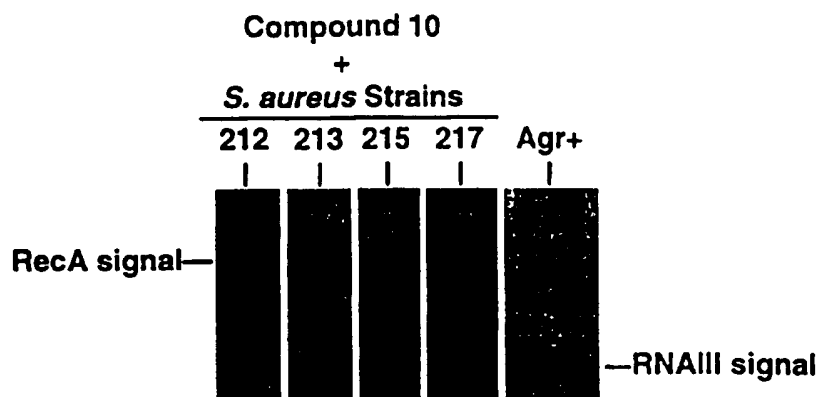
FIGURE 19.

FIGURE 20.



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶: A61K 31/00, 31/42, 31/165, 31/415, 31/44, 31/41, 31/19, 31/38, 31/35, 31/045, 31/365, 31/05	A3	(11) International Publication Number: WO 97/11690 (43) International Publication Date: 3 April 1997 (03.04.97)
(21) International Application Number: PCT/US96/15435 (22) International Filing Date: 25 September 1996 (25.09.96) (30) Priority Data: 60/004,626 29 September 1995 (29.09.95) US 08/672,215 25 June 1996 (25.06.96) US (71) Applicant: MICROCID PHARMACEUTICALS, INC. [US/US]; 850 Maude Avenue, Mountain View, CA 94043 (US). (72) Inventors: BAO, Ying; 1291 Vincent Drive #248, Sunnyvale, CA 94086 (US). BOGGS, Amy; 490 Sherwood Way #3, Menlo Park, CA 94025 (US). CONTAG, Pamela, R.; 6110 Bollinger Road, San Jose, CA 95129 (US). FEDERSPIEL, Nancy, A.; 1345 Altschul Avenue, Menlo Park, CA 94025 (US). HEBERT, Alan; 450 8th Avenue, Menlo Park, CA 94025 (US). HECKER, Scott; 16387 Englewood Avenue, Las Gatos, CA 95032 (US). MALOUIN, Francois; 18400 Overlook Road, #6, Los Gatos, CA 95030 (US). (74) Agents: WARBURG, Richard, J. et al.; Lyon & Lyon, Suite 4700, 633 West Fifth Street, Los Angeles, CA 90071 (US).		(81) Designated States: AU, CA, CU, DE, IL, JP, MX, NZ, European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE). Published <i>With international search report.</i> <i>Before the expiration of the time limit for amending the</i> <i>claims and to be republished in the event of the receipt of</i> <i>amendments.</i> (88) Date of publication of the international search report: 12 September 1997 (12.09.97)
(54) Title: INHIBITORS OF REGULATORY PATHWAYS		
(57) Abstract		
<p>Methods are provided for screening for potential inhibitors of bacterial, or other microbial, global pathogenesis gene regulators and other gene regulators. Methods are also provided for treating microbial (e.g., bacterial) infections using such inhibitors. Also included are pharmaceutical compositions containing such inhibitors. The screening methods involve detecting whether the activity of a global pathogenesis gene regulator is altered in the presence of a test compound.</p>		

* (Referred to in PCT Gazette No. 22/1997, Section II)

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INTERNATIONAL SEARCH REPORT

Inter national Application No
PCT/US 96/15435

A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 A61K31/00 A61K31/42 A61K31/165 A61K31/415 A61K31/44
A61K31/41 A61K31/19 A61K31/38 A61K31/35 A61K31/045
A61K31/365 A61K31/05

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	JOURNAL OF BACTERIOLOGY, vol. 176, no. 13, July 1994, pages 4168-4172, XP000645499 CHEUNG A L ET AL: "CLONING AND SEQUENCING OF SARA OF STAPHYLOCOCCUS AUREUS, A GENE REQUIRED FOR THE EXPRESSION OF AGR" see the whole document ---	1-7,9, 11-18, 20,22, 50,51, 79-81, 83,85-87
X,P	WO 96 10579 A (UNIV NEW YORK) 11 April 1996 see the whole document ---	1-22
X	WO 94 02586 A (AGRICULTURAL & FOOD RES ;PARK SIMON FEARON (GB)) 3 February 1994 see page 2, line 6 - line 8 ---	1
-/--		

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

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- *P* document published prior to the international filing date but later than the priority date claimed

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X document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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Date of the actual completion of the international search

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Date of mailing of the international search report

31.07.97

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Authorized officer

Trifilieff-Riolo, S

INTERNATIONAL SEARCH REPORT

Inter. Application No.

PCT/US 96/15435

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X,P	LV 10 915 B (BF ESSE SIA) 20 April 1996 see page 7; table 1 ---	1-23, 50-52, 79-87
X	JOURNAL OF BACTERIOLOGY, vol. 175, no. 24, December 1993, pages 7875-7879, XP000645497 HART M E ET AL: "THE EXTRACELLULAR PROTEIN REGULATOR (XPR) AFFECTS EXOPROTEIN AND AGR MRNA LEVELS IN STAPHYLOCOCCUS AUREUS" see the whole document ---	1-8, 11-19, 22,50, 51, 79-82, 85-87
X	INFECTION AND IMMUNITY, vol. 60, no. 8, August 1992, pages 3381-3388, XP000645259 REGASSA L B ET AL: "GLUCOSE AND NONMAINTAINED PH DECREASE EXPRESSION OF THE ACCESSORY GENE REGULATOR (AGR) IN STAPHYLOCOCCUS AUREUS" see the whole document ---	1-7, 11-18, 22,50, 51, 79-81, 85-87
X	CANADIAN JOURNAL OF MICROBIOLOGY, vol. 40, August 1994, pages 677-681, XP000645258 GIRAUDO A T ET AL: "CHARACTERIZATION OF A TN551-MUTANT OF STAPHYLOCOCCUS AUREUS DEFECTIVE IN THE PRODUCTION OF SEVERAL EXOPROTEINS" see the whole document ---	1-3,5,7, 10-14, 16,18, 21,22, 50,51, 79,80, 84,85
A	PHYTOCHEMISTRY, vol. 36, no. 4, 1994, XP000645959 ULUBELEN A ET AL: "TERPENOIDS FROM SALVIA SCLAREA" see the whole document ---	23,24, 52,53
X	P.N.A.S., vol. 90, 1993, pages 965-969, XP002035604 ROYCHOUDHURY ET AL: "inhibitors of 2-component signal transduction systems: inhibition of alginate gene activation in Pseudomonas aeruginosa" cited in the application see the whole document ---	88-90, 98,99
X	PATENT ABSTRACTS OF JAPAN vol. 095, no. 007, 31 August 1995 & JP 07 089924 A (SHIRATORI SEIYAKU KK), 4 April 1995, see abstract ---	61,62

-/--

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 96/15435

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	DE 31 08 067 A (BAYER AG) 16 September 1982 see page 19; example 3 ---	61,62
A	MED. PARAZITOL. PARAZIT. BOLEZNI, vol. 6, 1991, pages 52-53, XP002035605 MIKHAILITSYN ET AL: "search for new antiparasitic agents" see page 53; table 2 -----	40,41

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 96/ 15435

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claim(s) 1-49
is(are) directed to a method of treatment of the human/animal
body, the search has been carried out and based on the alleged
effects of the compound/composition.
2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such
an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

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1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all
searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment
of any additional fee.
3. ☒ As only some of the required additional search fees were timely paid by the applicant, this International Search Report
covers only those claims for which fees were paid, specifically claims Nos.:
Subjects 1 5 and 9
Please see next page for further information
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is
restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☒ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/210

- subject 1: claims 1 to 22 (all partially), 23, 24, 50-51 (both partially), 52, 53, 79-103 (all 24 partially): use of a compound of formula 1 or 1A to treat or prevent a bacterial infection, compositions comprising said compound, method of screening for such a compound, method of producing an antibacterial agent comprising this compound
- subject 2: claims 1 to 22 (all partially), 25, 26, 27, 50-51 (both partially), 54, 55, 56, 79-103 (all 24 partially): use, composition, method of screening and of producing an antibacterial agent as in subject 1 but relating to a compound of formula 2 or 2A (as far as not comprised in subject 1)
- subject 3: claims 1 to 22 (all partially), 28, 29, 50-51 (both partially), 57, 58, 79-103 (all 24 partially): use, composition, method of screening and of producing an antibacterial agent as in subject 1 but relating to a compound of formula 4 or 4A (as far as not comprised in subjects 1-2)
- subject 4: claims 1 to 22 (all partially), 30, 31, 50-51 (both partially), 59, 60, 79-103 (all 24 partially): use, composition, method of screening and of producing an antibacterial agent as in subject 1 but relating to a compound of formula 5 or 5A (as far as not comprised in subjects 1-3)
- subject 5: claims 1 to 22 (all partially), 32, 33, 50-51 (both partially), 61, 62, 79-103 (all 24 partially): use, composition, method of screening and of producing an antibacterial agent as in subject 1 but relating to a compound of formula 6 or 6A (as far as not comprised in subjects 1-4)
- subject 6: claims 1 to 22 (all partially), 34, 35, 50-51 (both partially), 63, 64, 79-103 (all 24 partially): use, composition, method of screening and of producing an antibacterial agent as in subject 1 but relating to a compound of formula 7 or 7A (as far as not comprised in subjects 1-5)
- subject 7: claims 1 to 22 (all partially), 36, 37, 50-51 (both partially), 65, 66, 79-103 (all 24 partially): use, composition, method of screening and of producing an antibacterial agent as in subject 1 but relating to a compound of formula 8 or 8A (as far as not comprised in subjects 1-6)
- subject 8: claims 1 to 22 (all partially), 38, 39, 50-51 (both partially), 67, 68, 79-103 (all 24 partially): use, composition, method of screening and of producing an antibacterial agent as in subject 1 but relating to a compound of formula 9 or 9A (as far as not comprised in subjects 1-7)
- subject 9: claims 1 to 22 (all partially), 40, 41, 50-51 (both partially), 69, 70, 79-103 (all 24 partially): use, composition, method of screening and of producing an antibacterial agent as in subject 1 but relating to a compound of formula 10 or 10A (as far as not comprised in subjects 1-8)
- subject 10: claims 1 to 22 (all partially), 42, 43, 50-51 (both partially), 71, 72, 79-103 (all 24 partially): use, composition, method of screening and of producing an antibacterial agent as in subject 1 but relating to a compound of formula 11 or 11A (as far as not comprised in subjects 1-9)
- subject 11: claims 1 to 22 (all partially), 44, 45, 50-51 (both partially), 73, 74, 79-103 (all 24 partially): use, composition, method of screening and of producing an antibacterial agent as in subject 1 but relating to a compound of formula 12 or 12A (as far as not comprised in subjects 1-10)

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subject 12: claims 1 to 22 (all partially), 46, 47, 50-51 (both partially), 75, 76, 79-103 (all 24 partially): use, composition, method of screening and of producing an antibacterial agent as in subject 1 but relating to a compound of formula 13 or 13A (as far as not comprised in subjects 1-11)

subject 13: claims 1 to 22 (all partially), 48, 49, 50-51 (both partially), 77, 78, 79-103 (all 24 partially): use, composition, method of screening and of producing an antibacterial agent as in subject 1 but relating to a compound of formula 14 or 14A (as far as not comprised in subjects 1-12)

INTERNATIONAL SEARCH REPORT

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PCT/US 96/15435

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